AF

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re application of

Hiroaki TAKAYAMA, et al.

Appln. No.: 09/214,155

Confirmation No.: 5866

Filed: December 29, 1998

For: VITAMIN D3 DERIVATIVE AND ITS PRODUCTION METHOD

Docket No: Q52816

Group Art Unit: 1616

Examiner: Sabiha N. Qazi

SUBMISSION OF APPEAL BRIEF

MAIL STOP APPEAL BRIEF - PATENTS
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Submitted herewith please find an Appeal Brief. A check for the statutory fee of $340.00 is attached. The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account. A duplicate copy of this paper is attached.

Respectfully submitted,

Susan J. Mack

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WASHINGTON OFFICE
23373
CUSTOMER NUMBER

Date: October 20, 2004

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
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Examiner: Sabiha N. Qazi

For: VITAMIN D3 DERIVATIVE AND ITS PRODUCTION METHOD

APPEAL BRIEF UNDER 37 C.F.R. § 41.37

MAIL STOP APPEAL BRIEF - PATENTS
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In accordance with the provisions of 37 C.F.R. § 41.37, Appellant submits the following:

The following comprises the Appellant's Brief on Appeal from the Office Action dated April 20, 2004, wherein claims 3 and 4 were finally rejected.

Appellant's Notice of Appeal was filed on August 20, 2004. Therefore, the present Appeal Brief is timely filed.

I. REAL PARTY IN INTEREST

The real party in interest is Teijin Limited, a Japanese Body Corporate.

II. RELATED APPEALS AND INTERFERENCES

There are no other appeals or interferences known to Appellants, the Appellants' legal representative, or Assignee which will directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal.

III. STATUS OF CLAIMS

Claims 3 and 4 are pending in the application, and their status is as follows:

Claims 3 are 4 are rejected and are the subject of this appeal.

IV. STATUS OF AMENDMENTS

On December 22, 2003, Appellants filed an Amendment Under 37 C.F.R. § 1.111 in response to the non-final Office Action mailed August 22, 2003. That Amendment was entered. There has not been an amendment filed in response to the April 20, 2004, final Office Action. Accordingly, the claims stand as presented in the Amendment filed December 22, 2003 (see attached Appendix).

V. SUMMARY OF THE CLAIMED SUBJECT MATTER

The claimed subject matter relates to novel 1, 25-dihydroxy-2-methylvitamin D_3 compounds having unexpectedly superior properties, and methods for producing the compounds. Specification, page 1, lines 8-10.

Claim 3 provides a 1,25-dihydroxy-2-methylvitamin D_3 compound, wherein the compound is (i) (20S)-1α, 25-dihydroxy-2β-methyl-3β-vitamin D_3; (ii) (20S)-1β, 25-dihydroxy-2β-methyl-3α -vitamin D_3; (iii) (20S)-1α, 25-dihydroxy-2α -methyl-3β-vitamin D_3; or (iv) (20S)-1α, 25-dihydroxy-2α -methyl-3α -vitamin D_3. Specification, page 6, lines 13, 16, 17, and 19.

Claim 4 provides a method for producing a vitamin D_3 compound described in claim 3, comprising reacting an exo-methylene compound of formula (II) (as defined in claim 4) with an eneyne compound of formula (III) (as defined in claim 4) in the presence of a palladium catalyst, and optionally removing the protecting group of the tri (C_1 to C_7 hydrocarbon) silyl) group. Specification, page 5, lines 10-30; page 6, lines 1-4; and pages 7-15.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

1. U.S. Application No. 10/069,481

Claim 3 was provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-3, 7 and 8 of copending U.S. Application No. 10/069,481.

At page 3 of the Office Action of April 20, 2004, the Examiner stated that although the conflicting claims are not identical, they are not patentably distict from each other because the 2-position of vitamin D represents a lower alkyl group and it is claimed in '481, where vitamin D_3 is a 2-substituted alkyl. The Examiner concluded that in the copending application and the present application the 2-substituted vitamin D_3 compounds which are claimed are considered obvious over each other.

2. U.S. Patent 5,945,410 and U.S. Patent 6,306,844 B1

Claim 3 was rejected under 35 U.S.C. §103(a) as being unpatentable over *DeLuca* et al., U.S. Patent 5,945,410 (US '410) and U.S. Patent 6,306,844 B1 (US '844).

According to the Examiner, both references teach 2-methyl and 2-alkyl 19-nor 20(S) vitamin D_3 compounds. The Examiner further asserted that US '410 teaches 1α, 25-dihydroxy-2α and 1α, 25-dihydroxy-2β methyl 19-nor vitamin D_3 (compound 12 and 13, example 1) and the biological activity of 2-methyl substituted 19-nor 1, 25-$(OH)_2D_3$ compounds and their 20-S isomers. The Examiner specifically referred to lines 36-67, in col. 15 and lines 1-37 in col. 16, where 2-methyl substitutions are said to produce surprisingly potent compounds.

3. Trost et al, J. Am. Chem. Soc., 114: 9836-45 (1992)

Claim 4 was rejected under 35 U.S.C. § 103(a) as being unpatentable over Trost et al, J. Am. Chem. Soc., 114: 9836-45 (1992) (Trost).

The Examiner's position was that Trost discloses a palladium-catalyzed alkylative cyclization of enyes for the synthesis of vitamin D derivatives. The Examiner asserted that the difference between the claimed invention and the disclosure of Trost is that in the present invention a different starting material is used wherein the starting compound of the claimed invention has a methyl group at the 4-position of the compound of formula III and Trost discloses an unsubstituted 4-position, but that both are enyes of formula III. Thus, the Examiner concluded that one of ordinary skill in the art would have been motivated to use the process of Trost in order to obtain the instant derivatives since the starting materials would be expected to react similarly.

VII. ARGUMENTS

1. The Provisional Obviousness-Type Double Patenting Rejection Of Claim 3 Over U.S. Application No. 10/069,481 Should Be Withdrawn.

Until cited by the Examiner, Appellants were not aware of the copending application and, furthermore, do not have access to the application or its claims.

Nonetheless, Appellants note that the present application claims benefit to May 2, 1997, whereas the cited application claims benefit to April 28, 1999 (information taken from corresponding international applications).

Accordingly, the obviousness-type double patenting rejection should be removed from the present application and maintained in the cited application, which was the later filed application.

Furthermore, the copending application cited by the Examiner is invalid and unpatentable over Appellants' corresponding International Publication No. WO 98/50353 published November 12, 1998. WO 98/50353 is believed to have been published more than one year prior to the actual U.S. filing date of U.S. Application No. 10/069,481, and therefore is legally effective art under 35 U.S.C. § 102 (b) and/or 103 (a). Accordingly, it is believed that the cited application will not issue.

Therefore, at a minimum, this provisional obviousness-type double patenting rejection should be held in abeyance until a decision on non-patentability is made in the cited application.

2. Claim 3 Is Patentable Under 35 U.S.C. § 103(a) Over DeLuca et al., U.S. Patent 5,945,410 (US '410) and U.S. Patent 6,306,844 B1 (US '844).

The cited US '844 patent is a CIP of U.S. Patent 6,127,559 (US '559).

The US '844 patent has a publication date of October 23, 2001, and a filing date of July 14, 2000, both of which are after Appellants' actual U.S. filing date of December 29, 1998. Similarly, the US '559 patent has a publication date of October 3, 2000, and a filing date of August 17, 1998, both of which are after Appellants' April 30, 1998, PCT filing date.

Therefore, the Examiner cannot rely upon any disclosure that is not also in the US '410 patent. In order to overcome this rejection, Appellants submitted two declarations under 37 C.F.R. § 1.131, establishing that the present inventors actually reduced to practice at least two species of the invention prior to the March 17, 1997 filing date of US '410. Therefore, the *DeLuca* references should be removed and the rejection withdrawn.

More specifically, an executed Rule 131 Declaration by Dr. Toshie Fujishima, one of the inventors, was submitted on December 18, 2002. Also submitted therewith as corroboration was an executed Rule 131 Declaration by Dr. Zhaopeng Liu.

In her declaration, Dr. Fujishima explaines that prior to March 17, 1997, she synthesized compound (68), (20S)-1α,25-dihydroxy-2β-methyl-3β-vitamin D_3 (Example 2, page 33 of the specification) and compound (72), (20S)-1α,25-dihydroxy-2α-methyl-3β-vitamin D_3 (Example 1, page 32 of the specification). The synthesis of compound (68) is documented in experimental note (1), Exhibit 1, and the synthesis of compound (72) is documented in experimental note (2), Exhibit 1, both of which are copies of pages from Dr. Fujishima's notebook. The entries on the pages are described in detail in the declaration. Dr. Fujishima also describes NMR spectra of compound (68) (Chart 1, Exhibit 1) and of compound (72) (Chart 2, Exhibit 1) from an analysis that was carried out prior to March 17, 1997. Dr. Fujishima testifies that she reviewed the NMR spectra prior to March 17, 1997 and identified the compounds as those compounds designated as compound (68) and compound

(72) in the present application. In addition Dr. Fujishima testifies that the vitamin D receptor affinity of compound (68) and of compound (72) was measured prior to March 17, 1997 (note 3 and Chart 3, Exhibit 1). Further, Dr. Fujishima testifies that she presented the results of the work to her colleagues prior to March 17, 1997. Handouts from the seminar were submitted with the declaration, and are explained in detail by Dr. Fujishima (Exhibit 2).

The declaration of Dr. Liu corroborates Dr. Fujishima's testimony. Specifically, Dr. Liu states that he remembers that Dr. Fujishima is the author of experimental notes (1), (2), and (3) (Exhibit 1) and that he recalls that Dr. Fujishima planned to present the results of these notes at a workshop. Dr. Liu testifies that to do so, Dr. Fujishima had to submit an abstract, which was due prior to March 17, 1997 (Exhibit 3). Dr. Liu also states that he was present when Dr. Fujishima presented the results of her work on compounds (68) and (72) in a group seminar held prior to March 17, 1997 (Exhibit 2). In his declaration, Dr. Liu describes in detail the handouts from the seminar and states that he understood that Dr. Fujishima had synthesized and confirmed the usefulness of compounds (68) and (72) prior to March 17, 1997. Dr. Liu also states that he understood that Dr. Fujishima had determined a detailed scheme for how to make compounds (68) and (72) prior to March 17, 1997.

As evidenced by the Fujishima and Liu declarations, two species within the claimed invention had been actually reduced to practice prior to the March 17, 1997 filing date of US '410. Accordingly, Appellants have antedated US '410.

For at least the reasons set forth above, claim 3 is patentable over US '410 and US '844.

3. Claim 4 Is Patentable Under 35 U.S.C. § 103(a) Over Trost M. Barry et al., J. Am. Chem. Soc., 114: 9836-45 (1992) (Trost).

The Examiner has not made a *prima facie* showing of obviousness. To establish a *prima facie* case of obviousness there must be (1) some suggestion or motivation within the reference or in the knowledge generally available to one of ordinary skill in the art to modify the reference; (2) a reasonable expectation of success; and (3) the reference relied upon by the Examiner must teach or suggest all of the claimed limitations. *See Hodesh v. Block Drug Co*, 786 F.2d 1136, 1153, n.5, 229 USPQ 182, 187, n.5 (Fed. Cir. 1986); *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 1438 (Fed. Cir. 1991); and *In re Royka*, 490 F.2d 981, 180 USPQ 580 (CCPA 1974).

In this case, there is no teaching or suggestion within the reference to modify the disclosed process such that one of ordinary skill in the art would have had a reasonable expectation of success in achieving the claimed 20(S) vitamin D_3 derivatives, having unexpectedly superior properties as shown in the specification on page 39 and in the Declarations under 37 C.F.R. § 1.132 submitted December 30, 1999, (the executed declaration was filed on August 1, 2000) and on November 13, 2001. It has been established that where a method for preparing purified isomers of compounds, such as the claimed 20(S) vitamin D_3 derivatives, is not taught or suggested by a reference, a *prima facie* case of obviousness has not been established. Appellants rely on the holding *of Emory University v. Glaxo Wellcome, Inc.* (44 USPQ2d 1407 (DC NGa 1997)) citing *In re Hoeksema* (158 USPQ 596, (CCPA 1968)) for the holding that "if the prior art of record fails to disclose or render obvious a method for making a claimed compound at the time the invention was made, it may not be legally concluded that the compound itself is in the possession of the public."

In addition, the Examiner failed to consider the claimed invention as a whole. The reference does not teach all of the limitations of the claimed invention, namely, the specifically recited 20(S) form vitamin D_3 derivative compounds. The fact that the reference may teach a similar process is not sufficient by itself to establish obviousness under the fact intensive inquiry required by 35 U.S.C. § 103. *See In re Ochiai*, 71 F.3d 1565, 37 USPQ2d 1127 (Fed. Cir. 1995) (reversing the Board of Appeals and the Examiner stating that both had used incorrect methodology in determining obviousness of a process for making a compound based upon a "general obviousness rule that a claim is obvious if prior art references disclose the same general process using similar starting materials" and stating that no such *per se* rule exists).

As in the case of *In re Ochiai*, the Examiner's rejection is based upon a general rule of obviousness that a process claim is obvious if the prior art references disclose the same general process using similar starting materials. The Examiner has maintained the position that the starting materials of the claimed invention are analogous to those taught by *Trost et al* because they are both enynes of formula III. As a basis for the rejection the Examiner stated and maintained: "[i]t has been held that application of an old process to a[n] analogous material to obtain a result consistent with the teachings of the art would have been obvious to one having ordinary skill." However such an analysis does not take into consideration the fact that the compounds of formula III of the claimed invention are different as well as the fact that the recited 20(S) vitamin D_3 derivative compounds are different and have been shown to possess unexpectedly superior properties over the closest specifically disclosed compounds.

Further, the compound of claim 4 is an intermediate compound rather than a starting material, as incorrectly stated by the Examiner, and the intermediate compound is essential for the claimed process with respect to the preparation of the specific 20(S) form vitamin D recited in the claims. The data showing unexpectedly superior results of the final product is sufficient to establish the patentability of the claimed process.

For at least the reasons set forth above, claim 4 is patentable over Trost.

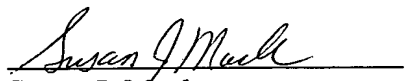
4. The Claims Do Not Stand And Fall Together

Claims 3 and 4 are directed to a compound and a method of making that compound. The claims are separately patentable (s*ee In re Ochiai*, 71 F.3d 1565).

Unless a check is submitted herewith for the fee required under 37 C.F.R. §41.37 and 1.17(c), please charge said fee to Deposit Account No. 19-4880.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

Respectfully submitted,



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Date: October 20, 2004

CLAIMS APPENDIX

CLAIMS ON APPEAL

Claim 3. A 1,25-dihydroxy-2-methylvitamin D_3 compound, wherein the compound is

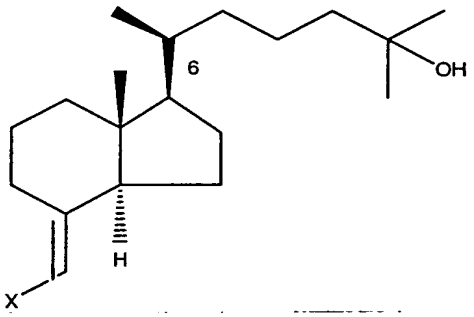
(i) (20S)-1α, 25-dihydroxy-2β-methyl-3β-vitamin D_3;

(ii) (20S)-1 β, 25-dihydroxy-2β-methyl-3α -vitamin D_3;

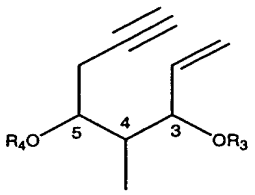
(iii) (20S)-1α, 25-dihydroxy-2α -methyl-3β-vitamin D_3; or

(iv) (20S)-1α, 25-dihydroxy-2α -methyl-3α -vitamin D_3.

Claim 4. A method for producing a vitamin D_3 compound described in claim 3, comprising reacting an exo-methylene compound of formula (II):



wherein X is a bromine atom or an iodine atom, with an eneyne compound of formula (III):



wherein R3 and R4 are each independently a hydrogen atom or a tri (C_1 to C_7 hydrocarbon) silyl) group in the presence of a palladium catalyst, and optionally removing the

protecting group of the tri (C_1 to C_7 hydrocarbon) silyl) group, and further wherein the vitamin D_3 compound is

(i) (20S)-1α, 25-dihydroxy-2β-methyl-3β-vitamin D_3;

(ii) (20S)-1β, 25-dihydroxy-2β-methyl-3α-vitamin D_3;

(iii) (20S)-1α, 25-dihydroxy-2α-methyl-3β-vitamin D_3; or

(iv) (20S)-1α, 25-dihydroxy-2α-methyl-3α-vitamin D_3.

EVIDENCE APPENDIX

Pursuant to 37 C.F.R. § 41.37(ix), submitted herewith are copies of any evidence submitted pursuant to 37 C.F.R. §§ 1.130, 1.131, or 1.132 or any other evidence entered by the Examiner and relied upon by Appellant in the appeal.

1. Executed Rule 131 Declaration by Dr. Toshie Fujushima, submitted December 18, 2002.

2. Executed Rule 131 Declaration by Dr. Zhaopeng Liu, also submitted December 18, 2002.

3. Executed Rule 132 Declaration by Dr. Ishizuka, submitted August 1, 2000.

4. Executed Rule 132 Declaration by Dr. Miura, submitted November 13, 2001.

5. Exhibit 1, submitted on December 18, 2002 with the executed Declarations of Drs. Toshie Fujushima and Zhaopeng Liu.

6. Exhibit 2, submitted on December 18, 2002 with the executed Declarations of Drs. Toshie Fujushima and Zhaopeng Liu.

7. Exhibit 3, submitted on December 18, 2002 with the executed Declarations of Drs. Toshie Fujushima and Zhaopeng Liu.

RELATED PROCEEDINGS APPENDIX

Submitted herewith are copies of decisions rendered by a court or the Board in any proceeding identified about in Section II pursuant to 37 C.F.R. § 41.37(c)(1)(ii).

NONE

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Hiroaki TAKAYAMA, et al.

Appln. No.: 09/214,155 Group Art Unit: 1616

Filed: December 29,1998 Examiner: Sabiha N. Qazi

For: VITAMIN D3 DERIVATIVE AND ITS PRODUCTION METHOD

DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Daishiro Miura, hereby declare and state:

THAT I am a citizen of Japan;

THAT I have received a Masters Degree in 1990 and a Ph.D. degree in 1999 from Hokkaido University;

THAT I am a member of several Japanese Scientific Societies related to research in toxicology, hematology, vitamin D and bone and mineral metabolism.

THAT I have been employed by Teijin Institute for Bio-Medical Research since 1990, in the Safety Research Department, where I have been involved in the toxicity study of pharmaceuticals and biological activities of Vitamin D_3.

I have thorough knowledge of the invention in the above-referenced patent application and I have read the final Office Action of June 12, 2001 issued in reference to the application. In response to the final Office Action, I submit herewith this Declaration comparing the potency of 20(S)-forms versus 20(R)-forms of Vitamin D derivatives and as evidence that the 20(S)-forms are significantly more potent than the 20(R)-forms in their ability to induce cellular differentiation.

Comparison of Activity for 20(S)- versus 20(R)-forms of Vitamin D derivatives for induction of HL-60 cell differentiation.

Materials and methods.

HL-60 cells were purchased from a cell bank (Japanese Cancer Research Resource Bank, Cell Number: JCRB 0085), and stored as a frozen stock to prevent any changes from occurring in the cell characteristics attributable to successive cultivation. Before the initiation of experiments, the cells were thawed, and passed by culturing. Cells which had been treated by successive culturing for one to six months, were used in the experiments. The successive culturing was carried out by culturing the cells in suspension, collecting the cell pellet by centrifugation, and diluting the cell pellet in fresh culture medium at a ratio of about 1/100 (1-2 x 10^5 cells/ml). The culture medium was RPMI-1640 containing 10% fetal bovine serum (FBS). Successively cultured cells were collected by centrifugation, and then were dispersed in culture medium at a concentration of 2 x 10^4 cells/ml. The suspended cells were seeded into a 24-well culture dish at 1 ml/well.

An ethanol solution containing the 20(S)-form compounds, (68), (71), (72) or (74), at concentrations ranging from 1 x 10^{-9} M to 1 x 10^{-6} M (for Table 1) or 1 x 10^{-7} M to 1 x 10^{-4} M (for Table 2) was added to each well at 1 µl/well. Further, regarding 1α,25-$(OH)_2D_3$, an ethanol solution containing 1 x 10^{-7} M to 1 x 10^{-4} M of the compound was added at 1 µl/well, and for the control, ethanol alone was added at 1 µl/well.

An ethanol solution containing the 20(R)-form compounds, (3), (4), (6) or (65), at concentrations ranging from 1x10^{-7} M to 1x10^{-4} M was added to each well at 1 µl/well. For the control, ethanol alone was added at 1 µl/well.

After culturing at 37°C for 4 days under a 5% CO_2 atmosphere, the cells were collected by centrifugation. Nitroblue tetrazolium (NBT) reducing activity was determined as follows: collected cells were suspended in a fresh culture medium, to which NBT and 12-O-tetradecanoylphorbol-13-acetate were added, so that the final concentrations were 0.1% and 100 nM, respectively. After mixing, the suspension was incubated at 37°C for 25 min. Then, cells were collected by centrifugation , resuspended in FBS and cytospin smears were prepared. After air-drying, smears were stained with Kernschtrot, and the ratio of blue stained to unstained cells (i.e., cells showing NBT reducing) was determined under an optical microscope.

Results.

The results are shown in the following Table1 (Comparison of activity for 20(S)-form compounds at concentrations ranging from 1×10^{-12} M to 1×10^{-9} M and 20(R)-form compounds at concentrations ranging from 1×10^{-10} M to 1×10^{-7} M) and Table 2 (Activity for 20(S)-form compounds at concentrations ranging from 1×10^{-10} M to 1×10^{-7} M). These results shown in Table 1 and Table 2 were obtained from independent two experiments.

Table 1.

Sample	Concentration (M)	% Cells showing NBT activity	Sample	Concentration (M)	% Cells showing NBT activity
Control		1.5	Control		5.8
$1\alpha,25\text{-}(OH)_2D_3$	10^{-10} 10^{-9} 10^{-8} 10^{-7}	4.3 ± 1.2 36.8 ± 2.0 86.1 ± 2.6 96.5 ± 1.0			
20(S)-form			**20(R)-form**		
Compound (68) [1]	10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7}	1.7 ± 0.3 2.8 ± 0.7 57.7 ± 5.0 95.7 ± 1.0 – –	Compound (65) [2]	10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7}	– – 1.8 ± 0.7 1.4 ± 0.8 32.0 ± 3.2 95.1 ± 2.8
Compound (71) [3]	10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7}	1.5 ± 0.8 1.8 ± 0.8 2.0 ± 1.0 40.5 ± 1.8 – –	Compound (3) [4]	10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7}	– – 1.6 ± 1.1 1.3 ± 0.5 1.8 ± 0.7 1.4 ± 0.2
Compound (74) [5]	10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7}	6.4 ± 1.1 17.0 ± 2.3 16.7 ± 1.1 96.4 ± 1.4 – –	Compound (6) [6]	10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7}	– – 1.9 ± 0.6 1.8 ± 0.2 12.9 ± 2.2 92.7 ± 2.9
Compound (72) [7]	10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7}	3.7 ± 0.8 94.4 ± 1.8 95.7 ±2.3 96.2 ±2.0 – –	Compound (4) [8]	10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7}	– – 2.3 ± 0.9 69.4 ± 3.9 93.9 ± 4.4 95.9 ± 1.1

[1] Example 2, page 33 of the specification.
[2] Page 40, line 1 of the specification.
[3] Example 5, page 36 of the specification.
[4] Page 39, line 30 of the specification.
[5] Example 7, page 37 of the specification.
[6] Page 39, line 33 of the specification.
[7] Example 1, page 32 of the specification.
[8] Page 39, line 31 of the specification.

Table 2.

Sample	Concentration (M)	% Cells showing NBT activity
Compound (68)	10^{-10}	90.8
	10^{-9}	98.3
	10^{-8}	98.0
	10^{-7}	99.5
Compound (71)	10^{-10}	22.0
	10^{-9}	47.1
	10^{-8}	97.5
	10^{-7}	97.7
Compound (74)	10^{-10}	52.1
	10^{-9}	95.4
	10^{-8}	97.7
	10^{-7}	98.7
Compound (72)	10^{-10}	93.7
	10^{-9}	97.9
	10^{-8}	98.6
	10^{-7}	98.6

Conclusions.

In a colorimetric cell assay which measures the ability of the compounds to induce differentiation of the HL-60 cell line vis-à-vis the reduction of nitroblue tetrazolium, the instant 20(S)-forms show excellent efficacy compared to the 20(R)-forms. Comparison of compound (68) with compound (65); compound (71) with compound (3); compound (74) with compound (6) and compound (72) with compound (4) reveals that the 20(S)-forms are substantially more potent, i.e., require logarithmically lower concentrations, in their ability to induce cell differentiation.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: November 7, 2001

Daishiro Miura

Daishiro Miura

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Hiroaki TAKAYAMA, et al.

Appln. No.: 09/214,155 Group Art Unit: 1616

Filed: December 29, ~~1999~~ 1998 Seiichi Ishizuka Examiner: Sabiha N. Qazi

For: VITAMIN D3 DERIVATIVE AND ITS PRODUCTION METHOD

DECLARATION UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Seiichi Ishizuka, hereby declare and state:

THAT I am a citizen of Japan;

THAT I have received a Masters Degree in 1974 and a Ph.D. degree in 1996 from Tohoku University;

THAT I am a member of several Japanese and American Scientific Societies related to research in vitamin D and bone and mineral metabolism, a referee for two scientific journals (Biochemistry and J. Nutritional Biochemistry), and the recipient of two scientific research awards (Vitamin D research award from Brown University (1995) and scientific research award from Japanese Society for Bone and Mineral Research (1997)).

THAT I have been employed by Teijin Institute for Bio-Medical Research since 1975, in the Department of Bone and Calcium Metabolism, where I have been involved in the study of the metabolism, biological activities and mechanism of actions of Vitamin D_3.

I have thorough knowledge of the invention in the above-referenced patent application and I have read the Office Action of June 30, 1999 issued in reference to the application. In response to the Office Action, I have conducted the following experiments to investigate the activity of 20R-forms for the vitamin D_3 derivatives disclosed in the reference cited by the Examiner for their effect on nitroblue tetrazolium reduction activity in the HL-60 cell line compared to the instant 20S-forms for the derivatives. A description of the experiments and the results obtained are the following.

Inventive Example 1 (20S-form of vitamin D_3 derivative)

Activity for 20S-forms of Vitamin D derivatives on induction of HL-60 cells to differentiate.

HL-60 cells were purchased from a cell bank (Japanese Cancer Research Resource Bank, Cell Number: JCRB 0085), and stored as a frozen stock to prevent any changes from occurring in the cell characteristics attributable to successive cultivation. Before the initiation of experiments, the cells were thawed, and passaged by culturing. Cells which had been treated by successive culturing for about one to six months, were used in the experiments. The successive culturing was carried out by culturing the cells in suspension, collecting the cell pellet by centrifugation, and diluting the cell pellet in fresh culture medium at a ratio of about 1/100 (1-2 x 10^5 cells/ml). The culture medium was RPMI-1640 containing 10% fetal bovine serum. Successively cultured cells were collected by centrifugation, and then were dispersed in culture medium at a concentration of 2 x 10^4 cells/ml. The suspended cells were seeded into a 24-well culture dish at 1 ml/well. An ethanol solution containing compounds 68, 71, 72 or 74 at

concentrations ranging from $1x10^{-9}$ M to $1x10^{-6}$ M was added to each well at 1 μl/well. Further, regarding 1 α, $25(OH)_2D_3$, an ethanol solution containing 1×10^{-7} M to 1×10^{-4} M of the compound was added at 1 μl/well, and for the control, ethanol alone was added at 1 μl/well. After culturing at 37°C for 4 days under a 5% CO_2 atmosphere, the cells were collected by centrifugation. Nitroblue tetrazolium (NBT) reduction activity was determined as follows: collected cells were suspended in a fresh culture medium, to which NBT and 12-O-tetradecanoylphorbol-13-acetate were added, so that the final concentrations were 0.1% and 100 nM, respectively. After mixing, the suspension was incubated at 37°C for 25 min, and the sample was removed for a cytospin centrifugation. After air drying, the cell pellet was stained with Kernschtrot, and the ratio of blue stained to unstained cells (i.e., cells showing NBT reduction) was determined under an optical microscope. The results are shown in the following Table.

Nitroblue Tetrazolium Reduction Activity in HL-60 Cells Treated with 20S-forms of Vitamin D Derivatives

Compound	Concentration (M)	% cells showing nitroblue tetrazolium reduction activity
Control		1.5
$1\alpha,25\text{-}(OH)_2D_3$	10^{-10}	4.3±1.2
	10^{-9}	36.8±2.0
	10^{-8}	86.1±2.6
	10^{-7}	96.5±1.0
Compound (68)	10^{-12}	1.7±0.3
	10^{-11}	2.8±0.7
	10^{-10}	57.7±5.0
	10^{-9}	95.7±1.0
Compound (71)	10^{-12}	1.5±0.8
	10^{-11}	1.8±0.8
	10^{-10}	2.0±1.0
	10^{-9}	40.5±1.8
Compound (74)	10^{-12}	6.4±1.1
	10^{-11}	17.0±2.3
	10^{-10}	16.7±1.1
	19^{-9}	96.4±1.4
Compound (72)	10^{-12}	3.7±0.8
	10^{-11}	94.4±1.8
	10^{-10}	95.7±2.3
	10^{-9}	96.2±2.0

Comparative Example 1 (20R form of vitamin D_3 derivatives)

Activity for 20R-forms of Vitamin D derivatives on induction of HL-60 cells to differentiate.

HL-60 cells were purchased from a cell bank (Japanese Cancer Research Resource Bank, Cell Number: JCRB 0085), and stored as a frozen stock to prevent any changes from occurring in the cell characteristics attributable to successive cultivation. Before the initiation of experiments, the cells were thawed, and passaged by culturing. Cells which had been treated by successive culturing for about one to six months, were used in the experiments. The successive culturing was carried out by culturing the cells in suspension, collecting the cell pellet by centrifugation, and diluting the cell pellet in fresh culture medium at a ratio of about 1/100 (1-2 x 10^5 cells/ml). The culture medium was RPMI-1640 containing 10% fetal bovine serum. Successively cultured cells were collected by centrifugation, and then were dispersed in culture medium at a concentration of 2 x 10^4 cells/ml. The suspended cells were seeded into a 24-well culture dish at 1 ml/well. An ethanol solution containing compounds 3, 4, 6 or 65 at concentrations ranging from $1x10^{-7}$ M to $1x10^{-3}$ M was added to each well at 1 µl/well. For the control, ethanol alone was added at 1 µl/well. After culturing at 37°C for 4 days under a 5% CO_2 atmosphere, the cells were collected by centrifugation. Nitroblue tetrazolium (NBT) reduction activity was determined as follows: collected cells were suspended in fresh culture medium to which NBT and 12-O-tetradecanoylphorbol-13-acetate were added, so that the final concentrations were 0.1% and 100 nM, respectively. After mixing, the suspension was incubated at 37°C for 25 min, and the sample was removed for cytospin centrifugation. After air drying, the cell pellet was stained with Kernschtrot, and the ratio of blue stained to unstained cells (i.e., cells showing NBT reduction) was determined under an optical microscope. The results are shown in the following Table.

Nitroblue Tetrazolium Reduction Activity in HL-60 Cells Treated with 20R-forms of Vitamin D Derivatives

Compound	Concentration (M)	% cells showing nitroblue tetrazolium reduction activity
Control		5.8
Compound (65)	10^{-8} 10^{-7} 10^{-6}	6.8 65.8 88.3
Compound (3)	10^{-8} 10^{-7} $3x10^{-7}$	6.8 11.4 80.9
Compound (6)	10^{-9} 10^{-8} 10^{-7} 10^{-6}	5.2 17.0 71.2 82.5
Compound (4)	10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6}	8.1 27.9 80.0 88.7 94.5

Conclusions.

In a colorimetric cell assay which measures the ability of the compounds to induce differentiation of the HL-60 cell line vis-à-vis the reduction of nitroblue tetrazolium, the instant 20S-forms show excellent efficacy compared to the 20R-forms. Comparison of compound (4) with compound (72); compound (65) with compound (68); compound (6) with compound (74) and compound (3) with compound (71) reveals that the 20S-forms are substantially more potent, i.e., require logarithmically lower concentrations, in their ability to induce cell differentiation.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: April 4, 2000

Seiichi Ishizuka

Seiichi Ishizuka

28007

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Hiroaki TAKAYAMA, et al.

Appln. No.: 09/214,155 Group Art Unit: 1616

Filed: December 29,1998 Examiner: Sabiha N. Qazi

For: VITAMIN D3 DERIVATIVE AND ITS PRODUCTION METHOD

FUJISHIMA DECLARATION UNDER 37 C.F.R. § 1.131

Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Toshie Fujishima, hereby declare and state:

THAT I am a citizen of Japan;

THAT I received a Masters Degree in 1993 and a Ph.D. degree in 1996 from Tokyo University;

THAT I am a member of several Japanese Scientific Societies related to research in organic chemistry and Vitamin D;

THAT I have been employed by Teikyo University, Faculty of Pharmaceutical Sciences since 1996, where I have been involved in the synthetic study of Vitamin D_3 in Professor TAKAYAMA's group.

I am one of the joint inventors of the invention claimed in the above-identified application.

I have a thorough knowledge of the invention in the above-identified patent application, and I have read the non-final Office Action of June 10, 2002 issued in reference to the application. In response to the non-final Office Action, I submit herewith this Declaration, which explains that I am the author of the experimental note of certain compounds claimed in the application and which explains my role in inventing the subject matter of the claims of the application.

Author of experimental notes

I am the author of experimental notes (1), (2), and (3). (*see* Exhibit 1).

My role in the invention claimed in the application

I synthesized compound (68) (Example 2, page 33 of the specification) prior to March 17, 1997, as described in experimental note (1) and compound (72) (Example 1, page 32 of the specification) prior to March 17, 1997, as described in experimental note (2). I ordered a NMR analysis of compound (68) and compound (72) from Instrument Analysis Center of Teikyo University, Faculty of Pharmaceutical Sciences, and the analysis was carried out prior to March 17, 1997 by Ms. Junko Shimode, a NMR operator at the center. Corresponding NMR spectra are shown in Exhibit 1, Chart 1 (Compound (68)) and Chart 2 (Compound (72)). I reviewed the NMR spectra prior to March 17, 1997, and I identified the compounds as those compounds designated compound (68) and compound (72) in the present application. I measured the Vitamin D receptor (VDR) affinity of compound (68) and compound (72) prior to March 17, 1997, as described in experimental note (3). Corresponding data from a liquid scintillation counter is shown in Exhibit 1, Chart 3. I presented the results of the work to my colleagues in one of Professor TAKAYAMA's group seminars prior to March 17, 1997. (*see* Exhibit 2)

Prior to March 17, 1997, I also submitted an abstract for a poster session at the Tenth Workshop on Vitamin D. (*see* Exhibit 3)

These activities are described in detail below with reference to the notebook pages and actual data. Because the text in my notebook and on the data is in Japanese, I have also included copies of the pages and data with English text added.

Exhibit 1 is a compilation of data and pages from my notebook number 2, which I kept in the ordinary course of my research. The entries are in my handwriting.

Note 1 is dated in the upper left-hand corner. The date is prior to March 17, 1997. This page shows synthesis of compound #346 from the protected precursor compound #345. Compound #346 is designated compound (68) in the above-identified application.

The upper left corner of the page also has the notation "#346." This means that I planned to make compound #346. The structure of synthesized compound #346 is shown in the upper right of the page. The structure is the same as that for the compound designated compound (68) in the specification of the above-identified application. The compound shown in the upper left of the page is the protected precursor. "TBS" stands for tert-butyldimethylsilyl. The arrow pointing from protected precursor compound #345 to synthesized compound #346 indicates that I planned to make synthesized compound #346 from protected precursor compound #345.

Below the structural formulas in a bracket is the notation "#345 work up," which means that I used compound #345 as a starting compound. I dissolved compound #345 in 1 ml of methanol (MeOH) and then added 11mg of CSA (Camphor sulfonic acid). The notations "20:30~" and "11:00," which appear to the right side of the open bracket mean that the reaction mixture was stirred from 20:30 (eight thirty PM), to 11:00 (eleven o'clock). The dates, which have been blocked out, indicate that the stirring time was about 38 ½ hours.

Below the open bracket, I indicate that the mixture was stirred at room temperature (rt) and then the methanol was evaporated from the reaction mixture. To the residue I added water and then extracted with EA (ethyl acetate). The extract was washed with brine, dried over $MgSO_4$, filtered, and evaporated to give compound #346.

Below this description I indicate that the crude product was purified by silica gel chromatography and further by HPLC (ODS (18)). I then ordered a ^1H NMR analysis of the compound.

Chart 1 is dated prior to March 17, 1997. Chart 1 is a ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound #346 i.e. compound (68) and its numerically outputted data. This data was assigned as compound #346 as follows: δ 0.55 (3 H, s), 0.85 (3 H, d, J = 6.4 Hz), 1.15 (3 H, d, J = 6.7 Hz), 1.21 (6 H, s), 2.42 (1 H, dd, J = 13.9, 4.9 Hz), 2.52 (1 H, br. d, J = 13.9 Hz), 2.82 (1 H, dd, J = 11.9, 4.0 Hz), 4.02 (2 H, m), 5.02 (1 H, t, J = 1.8 Hz), 5.37 (1 H, t, J = 1.8 Hz), 6.03 (1 H, d, J = 11.3 Hz), 6.35 (1 H, d, J = 11.3 Hz).

Note 2 is dated in the upper left-hand corner. The date is prior to March 17, 1997. This page shows synthesis of compound #344 from the protected precursor compound #343. Compound #344 is designated compound (72) in the above-identified application.

The upper left corner of the page also has the notation "#344." This means that I planned to make compound #344. The structure of compound #344 is shown in the upper right of the page. The structure is the same as that for the compound designated compound (72) in the specification of the above-identified application. The compound shown in the upper left of the page is the protected precursor. "TBS" stands for tert-butyldimethylsilyl. The arrow pointing from compound #344 to compound #343 indicates that I planned to make compound #344 from compound #343.

In a bracket below the structural formulas is the notation "#343 work up," which means that I used compound #343 as a starting compound. I dissolved compound #343 in 1 ml of methanol (MeOH) and then added 11 mg of CSA (camphor sulfonic acid).

Below the open bracket, I indicate that the mixture was stirred from 14:20 (two twenty PM) to 9:00 (nine o'clock), the next day under Ar gas at room temperature (rt), and then the methanol was distilled away from the reaction mixture. To the residue I added water and then extracted with EA (ethyl acetate). The extract was washed with brine, dried over $MgSO_4$, dehydrated, filtered, and evaporated to give compound #344.

Below this description I indicate that the crude product was purified by silica gel chromatography and further by HPLC (ODS (18)). "ϕ 0.9 cm 10 cm height" means silica gel column size (0.9 cm diameter and 10 cm length). "EA : n-hex = 1:1" indicates the solvent system for the chromatography (ethyl acetate : n-hexane = 1 : 1).

Drawings at the bottom of the page are illustrations of TLC (thin layer chromatography). "EA : n-hex = 1:1" means a solvent system of the TLC (ethyl acetate : n-hexane = 1 : 1). "SM" and "RM" mean starting material and reaction mixture. Circles in the illustrations show spots on TLC. TLC is a typical monitor system of organic reaction because the spots show compound(s) in analyzed material. A position difference between the spot labeled "SM" and the spot labeled "RM" means that the compound(s) in the "SM" and "RM" spots were different and the reaction was complete.

I then ordered a ^{1}H NMR analysis of the compound.

Chart 2 is dated prior to March 17, 1997. Chart 2 is a ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound #344 i.e. compound (72) and its numerically outputted data. This data assigned as compound #344 as follows: δ 0.53 (3 H, s), 0.85 (3 H, d, J = 6.7 Hz), 1.08 (3 H, d, J = 6.8 Hz), 1.21 (6 H, s), 2.23 (1 H, dd, J = 13.4, 7.9 Hz), 2.67 (1 H, dd, J = 13.4, 4.0 Hz), 2.83 (1 H, m), 3.83 (1 H, ddd, J = 7.9, 4.4, 4.0 Hz), 4.29 (1 H, d, J = 3.3 Hz), 5.01 (1 H, d, J = 1.8 Hz), 5.28 (1 H, m), 6.01 (1 H, d, J = 11.3 Hz), 6.39 (1 H, d, J = 11.3 Hz).

Note 3 is dated prior to March 17, 1997. The 1st through 3rd pages show the employed protocol for VDR binding assay.

The 1st page shows the 1st to the 8th steps of the protocol. The 1st, I made phosphate buffer and kept it at 4 °C. The 2nd, I prepared diluted solutions of 1α,25(OH)$_2$VD$_3$, compound #344 and compound #346. The 3rd, I prepared [26,27-methyl^{3}H]1α,25(OH)$_2$VD$_3$ solution, took 100 μl of the solution and evaporated, then I added 6.25 ml of Japanese pharmacopeia grade ethanol to the solution. The 4th, I

poured 50 µl of the sample in Japanese pharmacopeia grade ethanol (made in the 2nd step) into disposable culture tubes (12 x 75 mm, product of IWAKI) in order of concentration from thin to dense by dispenser (for example from tube number 14, 28 to 1, 15). Into tubes from number 85 to 96 was poured Japanese pharmacopeia grade ethanol only. The 5th, I made a receptor solution (lot 110431, product of YAMASA). I poured 5 ml of phosphate-potassium buffer (made in the 1st step) into a vessel containing the thymus receptor, dissolved the receptor gently, added further 50 ml of the buffer and stirred gently. The 6th, I added 500 µl of the receptor solution to each tube except blank tubes (tube number 89, 90, 91, 92), and I added 500 µl of the buffer solution to blank tubes. The 7th, I stirred solutions in the tubes (made in the 6th step) by vortex without foaming. The 8th, I pre-incubated the solutions in the tubes at room temperature (approximately 22 °C) for 1 hour (13:40 - 14:40). The tops of the tubes were sealed with plastic wrap and aluminum foil.

The 2nd page shows the 9th to the 11th steps of the protocol. These steps were carried out in RI (radio isotope) room. The 9th, I added 50 µl of the radioactive solution (made in the 3rd step) to each tube by dispenser. In the case of radioactivity only count (tube number 97, 98, 99, 100), radioactive solution was added to the vial. The 10th, I stirred the solution in the tubes by vortex without foaming. The 11th, I sealed the top of the tubes with plastic wrap, put the tubes in a 4 °C refrigerator in the RI room, and stood the tubes overnight from 15:10 (ten after three PM). Then I added 10 ml of ACS-II (Aqueous counting scintillant, product of Amersham) to the tubes and measured radioactivity count for 1 min by Aloka A machine (Scintillation counter). Then I stood the tubes at rt, and the next day I measured the radioactivity count for 2 min with other samples. The data measured for 1 min is shown at the middle of this page. "97 16217.7 dpm" means radioactivity count of tube number 97 was 16217.7 disintegrations per minute. "Average 16370 dpm" means calculated average value of tubes 97, 98, 99, 100 counts which are radioactive only solution. "Average 45 dpm" means calculated average value of tubes 101, 102, 103, 104

counts which are blank solution. In the parenthesis at the bottom of this page there is a calculation of the [26,27-methyl^{3}H]1α,25(OH)$_2$VD$_3$ weight per tube. 16370 dpm is 16370/60 = 273 dps (disintegrations per second), 273 dps is 273 Bq (dps unit equals Bq - becquerel- unit). The radioactivity of the [26,27-methyl^{3}H]1α,25(OH)$_2$VD$_3$ solution used in the 3rd step was 11.4 GBq/mg, (= 11.4 Bq/pg,), so the amount of [26,27-methyl^{3}H]1α,25(OH)$_2$VD$_3$ was 273/11.4 = 24 pg / tube.

The 3rd page shows from the 12th to the 17th steps of the protocol. I carried out these steps the day after the previous steps were done. The 12th, I took the previous day's samples from the refrigerator in the RI room and added 200 µl of DCC (dextran coated charcoal) solution (lot M602 product of YAMASA) to each tube by dispenser except total count tubes (tube number 93, 94, 95, 96). And I added 200 µl of buffer (made in the 1st step) to each of the total count tubes. The 13th, I stirred the tubes by vortex. The 14th, I stood the tubes for 30 min (9:50 - 10:20) at 4 °C. The 15th, the tubes were centrifuged at 3000 rpm for 10 min (10:30 - 10:40) at 0 °C. The 16th, I transferred 500 µl of supernatant of each tube to 20 ml WERATON vial in concentration order from thin to dense (for example from tube number 1 to 14 by same pipetter tip, changed pipetter tip, then from tube number 15 to 28). At that time the vials were put on tray with ice. The 17th, I added 9.5 ml of ACS-II to each of the tubes, shook them and measured its radioactivity count (2min) by Aloka A machine.

The 4th page shows a graph of the radioactivity count (Y-axis) of compounds #346, #344 and 1α,25(OH)$_2$VD$_3$ (control) solutions at various concentration (X-axis) at the 11th step of the assay protocol. Closed circles (VD) and closed triangle (VD-2) show 1α,25(OH)$_2$VD$_3$ values, open circles (344) and crosses (344-2) show #344 compound values and closed diamonds (346) show #346 compound values. These data were measured by Aloka C machine for 1 min. I measured the data from tube number 1 to 70.

The 5th page shows a table of the radioactivity count (dpm) of the compounds #346, #344 and 1α,25(OH)$_2$VD$_3$ (control) solutions at various concentrations. The first column shows the compound's

concentration, the 2nd and 3rd columns show the radioactivity counts of $1\alpha,25(OH)_2VD_3$ (duplicate experiment), the 4th and 5th columns show the radioactivity counts of #344 compound (duplicate experiment) and the 6th and 7th columns show the radioactivity counts of #346 compound (duplicate experiment). The small numerals on the line show tube number.

The 6th page shows a table of the radioactivity count of control solutions. "0" in the 1st column means the concentration of the compound is 0, "blank" right below "0" means buffer solution (made in the 1st step of the assay protocol) only, "total count" means radioactive solution (made in the 3rd step of the assay protocol) in the assay tube, "added amount" means added radioactive solution (made in the 3rd step of the assay protocol) and "blank" right below "added amount" means empty tubes. These data was taken by 4 tubes each. The most right column shows the average of the 4 values. The sentence at the middle of this page describes how to calculate the Bound [%] values. The "Bound [%]" means binding percentage of radioactive ligand ([26,27-methyl^{3}H]1α,25(OH)2VD3) to Vitamin D receptor.

The 7th page shows a graph and a table of the radioactivity count of compounds #346, #344 and $1\alpha,25(OH)_2VD_3$ (control) solutions at various concentrations at the last step of the assay protocol. The graph is shown in the same format as the graph on the 4th page, and the table is shown in the same format as the table on the 5th page.

The 8th page shows the method for calculating the binding ratio from the radioactivity data. First I calculated the average values of "blank" and "0" (218 and 2980). Then I described how to calculate the Bound [%] values. In the open bracket, I calculated the radioactive ligand ([26,27-methyl^{3}H]$1\alpha,25(OH)_2VD_3$) amount in the assay tubes from "total count" values (19 pg / tube). Then I calculated added radioactive ligand from "added amount" values (24 pg / tube). Then I speculated as to why there was a difference between the values for the "total count" and the "added amount."

The 9th page shows a graph and a table of the binding ratio of compounds #346, #344 and $1\alpha,25(OH)_2VD_3$ (control) at various concentrations. The binding ratio was calculated by the method described in the 8th page. In the graph, the concentration value (X-axis) of the compounds #346, #344 and $1\alpha,25(OH)_2VD_3$ at 50% bound (Y-axis) are 10-20 pg, 1-2 pg and 20 pg. These data show the binding affinity of compound #344 / #346 is 10-12 / 1-1.8 fold stronger than that of $1\alpha,25(OH)_2VD_3$.

Chart 3 is dated prior to March 17, 1997. Chart 3 is an output of the liquid scintillation counter mentioned on the 3rd page of Note 3. The data were transferred to the 5th and 6th pages of Note 3. The upper half side of the 1st page shows the settings of the scintillation counter. The bottom side of the 1st page and 2nd page shows raw data. The most left column shows tube number, the 3rd column shows the period of measuring (2 min) and the 5th column shows the radioactivity count (unit is dpm).

Exhibit 2 is a set of copies of handouts that I distributed at the group seminar referred to above. The handouts consist of 2 parts. As described at the top of the 1st page of the handout the first part relates to Synthesis of 2-methyl-20epi $1\alpha,25(OH)_2VD_3$.

Page 1 shows the synthesis scheme for compounds #346 and #344, i.e. compounds (68) and (72) respectively. The 1st arrow pointing from vitamin D_2 to compound 1 indicates that I made compound 1 from vitamin D_2 by treatment of vitamin D_2 with O_3 (Ozone) and $NaBH_4$ (Sodium borohydride). The 2nd arrow pointing from compound 1 to compound 2 indicates that I made compound 2 from compound 1 by treatment of compound 1 with TsCl (Tosyl chrolide) in pyridine (Yield 86%). The 3rd arrow pointing from compound 2 to compound 3 indicates that I made compound 3 from compound 2 by treatment of compound 2 with TBSOTf (tert-Butyldimethylsilyl triflate) in 2,6-lutidine (Yield 96%). The 4th arrow pointing from compound 3 to compound 4 indicates that I made compound 4 from compound 3 by treatment of compound 3 with DMSO (Dimethylsulphoxide) and $NaHCO_3$ (Sodium bicarbonate) (Yield 76%). The 5th arrow pointing from compound 4 to compound in parenthesis indicates that I made the

compound in parenthesis from compound 4 by treatment of compound 4 with n-Bu_4NOH (normal-Tetrabutylammonium hydroxide) in CH_2Cl_2 (Dichloromethane) and H_2O (water). The 6th arrow pointing from the compound in parenthesis to compound 5 indicates that I made compound 5 from the compound in parenthesis by treatment of the compound in parenthesis with $NaBH_4$ (Sodium borohydride) and stereoisomer separation by silica gel column chromatography (Yield 45% in 2 steps). The 7th arrow pointing from compound 5 to compound 6 indicates that I made compound 6 from compound 5 by treatment of compound 5 with TsCl (Tosyl chrolide) in pyridine (Yield 93%). The 8th arrow pointing from compound 6 to compound 7 indicates that I made compound 7 from compound 6 by treatment of compound 6 with NaI (Sodium iodide) in DMF (Dimethyl formamide) (Yield 92%). The 9th arrow pointing from compound 7 to compound 8 indicates that I made compound 8 from compound 7 and the compound described above the arrow by treatment of both compounds with n-BuLi (normal-Butyl lithium) and HMPA (Hexamethylphosphoramide) in THF (Tetrahydrofuran) (Yield 72% with recovered 28% of starting compound 7. The yield of this step was increased when using distilled HMPA --- 99%). The 10th arrow pointing from compound 8 to compound 9 indicates that I made compound 9 from compound 8 by treatment of compound 8 with Na-Hg (sodium-mercury amalgam) in THF (Tetrahydrofuran) (Yield 64%). The 11th arrow pointing from compound 9 to compound 10 indicates that I made compound 10 from compound 9 by treatment of compound 9 with TsOH (p-Toluenesulfonic acid) (Yield 85%). The 12th arrow pointing from compound 10 to compound 11 indicates that I made compound 11 from compound 10 by treatment of compound 10 with TPAP (Tetrapropylammonium perruthenate), NMO (N-methylmorpholine) and 4ÅMS (molecular sieves 4 angstrom) (Yield 87%). The 13th arrow pointing from compound 11 to compound 12 indicates that I made compound 12 from compound 11 by treatment of compound 11 with $Ph_3P(+)CH_2Br \cdot Br(-)$ ((Bromomethyl)triphenylphosphonium bromide) and NaHDMS (Sodium hexamethyldisilazide) (Yield

FUJISHIMA DECLARATION UNDER 37 C.F.R.§ 1.131
U.S. Appln. No. 09/214,155

The abstract confirms that I had designed and synthesized the 2-methyl-20-epi analogues of 1α,25-dihydroxyvitamin D_3 and had determined the activity of the analogues. In the abstract, compounds (1) and (2) correspond to compounds (72) and (68) in the specification of the above-identified application.

The instructions confirm that the abstract had to be submitted prior to March 17, 1997.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: December 9, 2002

Toshie Fujishima
Toshie Fujishima

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Hiroaki TAKAYAMA, et al.

Appln. No.: 09/214,155 Group Art Unit: 1616

Filed: December 29,1998 Examiner: Sabiha N. Qazi

For: VITAMIN D3 DERIVATIVE AND ITS PRODUCTION METHOD

LIU DECLARATION UNDER 37 C.F.R. § 1.131

Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Zhaopeng Liu, hereby declare and state:

THAT I am a citizen of P. R. China;

THAT I received a Masters Degree in 1990 from Shandong Medical University, P. R. China and a Ph.D. degree in 2001 from Toyama Medical and Pharmaceutical University, Japan;

THAT I am a member of Japanese Scientific Society related to research in organic chemistry;

THAT I have belonged to Teikyo University, Faculty of Pharmaceutical Sciences, in 1995-1998 as a research fellow, where I have been involved in the synthetic study of Vitamin D_3 in Professor TAKAYAMA's group;

THAT I have been employed by Foreigners Brain Company since 2001, where I have been involved in the synthetic research on new drug candidates.

I have thorough knowledge of the invention in the above-identified patent application, and I have read the non-final Office Action of June 10, 2002 issued in reference to the application. In response to the non-final Office Action, I submit herewith this Declaration, explaining what I observed pertaining to the reduction to practice of the invention claimed in the above-identified application.

Dr. Fujishima was my colleague when I worked in professor TAKAYAMA's group as a research fellow from 1995 to 1998. We understood each other's work very well through daily discussions and regular group seminars. At the time, one of the main research projects of Professor TAKAYAMA's group was the synthetic and biological study of novel 2-substituted Vitamin D_3. Dr.

Fujishima and I were core members of the project. The compounds in the application are a result of that project.

I recall certain incidents which lead me to remember the author and date of experimental notes (1), (2) and (3) very clearly: Dr. Fujishima planned to present the research results of the 2-substituted Vitamin D_3 project at the Tenth Workshop on Vitamin D, so she had to complete the synthesis and measurement of Vitamin D receptor affinity of the compounds in the application prior to the deadline for submission of presentation abstracts, which was prior to March 17, 1997. Dr. Fujishima presented the results of her work on compounds (68) and (72) in a group seminar held prior to March 17, 1997. Exhibit 2, submitted herewith, comprises copies of the handout that Dr. Fujishima distributed at that seminar. I remember seeing and understanding the handout at the time of the seminar.

Page 1 shows a synthesis scheme for compounds #346 and #344, i.e. compounds (68) and (72) respectively, that had been carried out by Dr. Fujishima or under her supervision. The 1st arrow pointing from vitamin D_2 to compound 1 indicates that compound 1 was made from vitamin D_2 by treatment of vitamin D_2 with O_3 (Ozone) and $NaBH_4$ (Sodium borohydride). The 2nd arrow pointing from compound 1 to compound 2 indicates that compound 2 was made from compound 1 by treatment of compound 1 with TsCl (Tosyl chrolide) in pyridine (Yield 86%). The 3rd arrow pointing from compound 2 to compound 3 indicates that compound 3 was made from compound 2 by treatment of compound 2 with TBSOTf (tert-Butyldimethylsilyl triflate) in 2,6-lutidine (Yield 96%). The 4th arrow pointing from compound 3 to compound 4 indicates that compound 4 was made from compound 3 by treatment of compound 3 with DMSO (Dimethylsulphoxide) and $NaHCO_3$ (Sodium bicarbonate) (Yield 76%). The 5th arrow pointing from compound 4 to the compound in parenthesis indicates that the compound in parenthesis was made from compound 4 by treatment of compound 4 with n-Bu_4NOH (normal-Tetrabutylammonium hydroxide) in CH_2Cl_2 (Dichloromethane) and H_2O (water). The 6th arrow pointing from the compound in parenthesis to compound 5 indicates that compound 5 was made from the compound in parenthesis by treatment of the compound in parenthesis with $NaBH_4$ (Sodium borohydride) and stereoisomer separation by silica gel column chromatography (Yield 45% in 2 steps). The 7th arrow pointing from compound 5 to compound 6 indicates that

compound 6 was made from compound 5 by treatment of compound 5 with TsCl (Tosyl chrolide) in pyridine (Yield 93%). The 8th arrow pointing from compound 6 to compound 7 indicates that compound 7 was made from compound 6 by treatment of compound 6 with NaI (Sodium iodide) in DMF (Dimethyl formamide) (Yield 92%). The 9th arrow pointing from compound 7 to compound 8 indicates that compound 8 was made from compound 7 and the compound described above the arrow by treatment of both compounds with n-BuLi (normal-Butyl lithium) and HMPA (Hexamethylphosphoramide) in THF (Tetrahydrofuran) (Yield 72% with recovered 28% of starting compound 7. The yield of this step was increased when using distilled HMPA --- 99%). The 10th arrow pointing from compound 8 to compound 9 indicates that compound 9 was made from compound 8 by treatment of compound 8 with Na-Hg (sodium-mercury amalgam) in THF (Tetrahydrofuran) (Yield 64%). The 11th arrow pointing from compound 9 to compound 10 indicates that compound 10 was made from compound 9 by treatment of compound 9 with TsOH (p-Toluenesulfonic acid) (Yield 85%). The 12th arrow pointing from compound 10 to compound 11 indicates that compound 11 was made from compound 10 by treatment of compound 10 with TPAP (Tetrapropylammonium perruthenate), NMO (N-methylmorpholine) and 4ÅMS (molecular sieves 4 angstrom) (Yield 87%). The 13th arrow pointing from compound 11 to compound 12 indicates that compound 12 was made from compound 11 by treatment of compound 11 with $Ph_3P(+)CH_2Br \cdot Br(-)$ ((Bromomethyl)triphenylphosphonium bromide) and NaHDMS (Sodium hexamethyldisilazide) (Yield 57%). The 14th and 15th arrows pointing from compound 12 to 20epi-Ds (compound #344) and 20epi-Aa (compound #346) indicate that 20epi-Ds and 20epi-Aa were made from compound 12 and a TBS protected compound (described in the upper of the page 5 and 6 of the Exhibit 2 (Ref.1)) by treatment of both compounds with $(dba)_3Pd_2CHCl_3$, (Tris(dibenzylideneacetone)dipalladium(0)-chloroform adduct), Ph_3P (Triphenylphosphine) and Et_3N (Triethylamine) in toluene, and then treatment of the resultant compounds with CSA (Camphor sulfonic acid) in MeOH (methanol).

Therefor, I understood that Dr. Fujishima synthesized compounds (68) and (72) prior to March 17, 1997.

Page 2 shows the method for the VDR binding assay. The resultant binding curves for compounds #346, # 344 and 1-α, 25-$(OH)_2$-VD_3 (control) are also shown. The resultant binding curves of compounds #346, # 344 and 1α,25$(OH)_2VD_3$ (control) are also shown. The upper half of the page shows the method for VDR binding assay which has the same content as the description in the 1st through 3rd pages of Note 3. The bottom half side of the page shows the resultant binding curves which is the same graph described on the 9th page of Note 3.

Thus, I understood that Dr. Fujishima had confirmed the usefulness of compounds (68) and (72) prior to March 17, 1997.

Pages 3 through 6 show the detailed process for synthesizing compounds #346 and #344 from compound #7 as described in scheme 1 on page 1, accompanied by NMR and MS data of each intermediate compound. As for compounds #346 and #344 i.e. compounds (68) and (72), UV data are also shown. Page 3 shows the detailed process for synthesizing compound 8 from compound 7 and compound 9 from compound 8. Page 4 shows the detailed process for synthesizing compound 10 from compound 9, compound 11 from compound 10 and compound 12 from compound 11. Page 5 shows the detailed process for synthesizing 20epiDs (compound #344) from compound 12 and a TBS protected compound. Page 6 shows the detailed process for synthesizing 20epiAa (compound #346) from compound 12 and a TBS protected compound.

From this I understood that Dr. Fujishima had determined a detailed scheme for how to make compounds (68) and (72) and had identified them prior to March 17, 1997.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 2002.12.10

Zhaopeng Liu

Zhaopeng Liu

VERIFICATION OF TRANSLATION

I, Kazuya Takenouchi of c/o Teijin Limited, Tokyo Research Center, 3-2, Asahigaoka 4-chome, Hino-shi, TOKYO, 191-0065 JAPAN, hereby certify that to the best of my knowledge and belief the attached English translation is a true translation, made by me and for which I accept responsibility, of the U.S. patent application number 09/214155, filed in the United States on November 29, 1998.

This 26th day of September, 2002

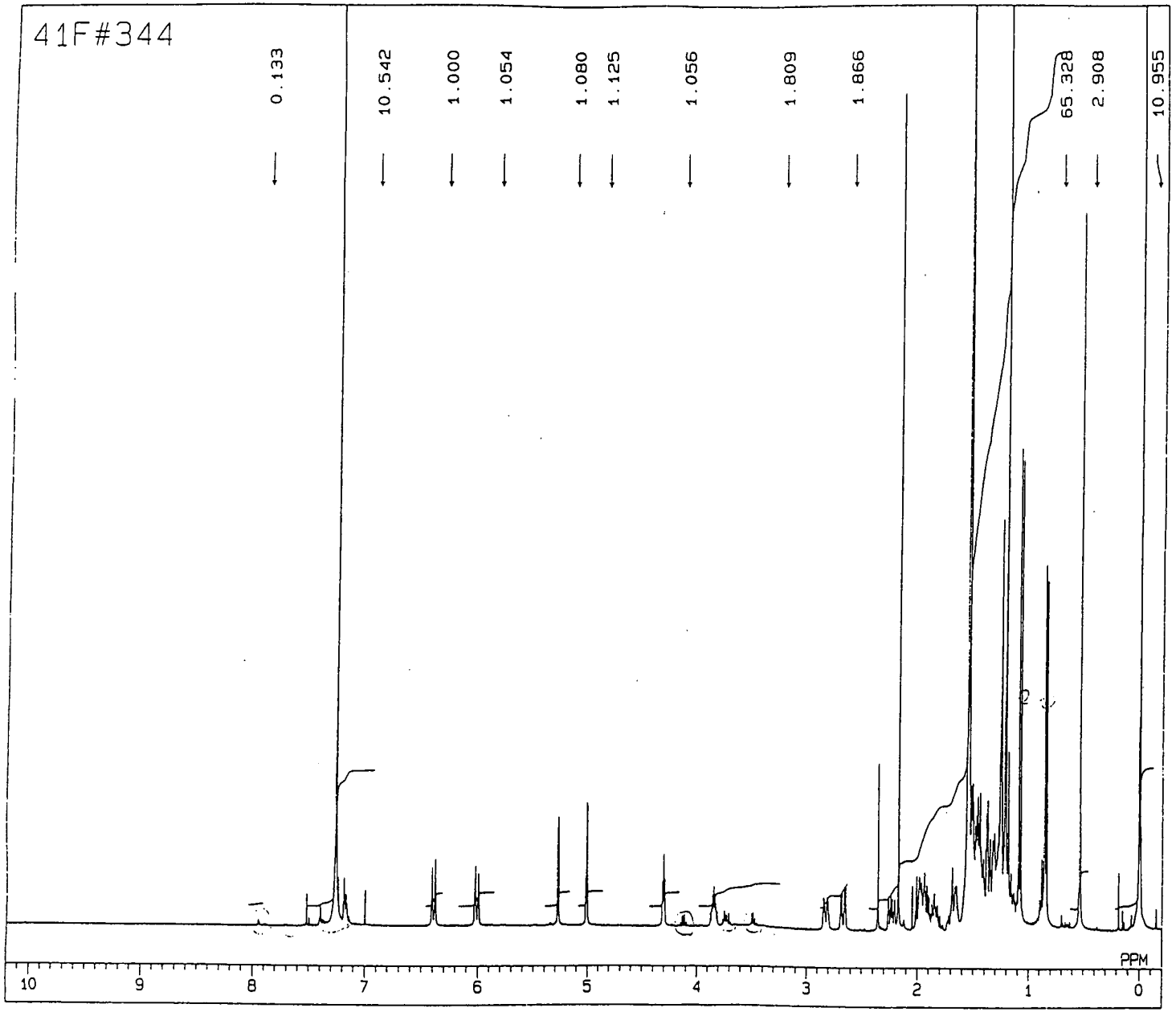
Kazuya Takenouchi

Exhibit 1
Chart 2, p. 2

PEAK	173
MXINT	996134500
RESOL	0.3100000 Hz
RESOL	0.0007754 PPM
EXREF	0.0000000 PPM
OBS	-2398.68 Hz
ABOBS	399784.9000000 KHz
NGAIN	22
COMNT	41P#344

NO.	PPM	INT(%)	FREQ(Hz)	POSITION	BAR GRAPH
1	7.51981	0.51055	3006.29	6201	
2	7.49843	0.13021	2997.74	6229	
3	7.40836	0.12475	2961.73	6347	
4	7.40530	0.13811	2960.51	6351	
5	7.40225	0.14221	2959.29	6355	
6	7.39614	0.26281	2956.85	6363	
7	7.39385	0.30828	2955.93	6366	
8	7.27401	0.81013	2908.02	6523	
9	7.26103	100.00000	2902.83	6540	++++++++++++++++++++
10	7.24347	0.52661	2895.81	6563	
11	7.23813	0.77035	2893.68	6570	
12	7.18469	0.76414	2872.31	6640	
13	7.17782	0.42273	2869.57	6649	
14	7.16790	0.44624	2865.60	6662	
15	7.16637	0.50551	2864.99	6664	
16	7.16485	0.47254	2864.38	6666	
17	7.15798	0.35221	2861.63	6675	
18	7.14042	0.13947	2854.61	6698	
19	6.99691	0.56436	2797.24	6886	
20	6.40531	0.93125	2560.73	7661	
21	6.37706	1.07192	2549.46	7698	
22	6.02516	0.95532	2408.75	8159	
23	5.99691	0.83247	2397.46	8196	11.29
24	5.28089	1.51383	2111.21	9134	
25	5.27860	1.69251	2110.29	9137	
26	5.27554	1.73099	2109.07	9141	
27	5.27325	1.62102	2108.15	9144	
28	5.01677	0.75333	2005.62	9480	
29	5.01142	1.94912	2003.48	9487	
30	5.00684	1.89288	2001.65	9493	
31	4.31372	0.71248	1724.35	10401	
32	4.30303	1.15135	1720.28	10415	
33	4.29311	0.75016	1716.31	10428	3.97
34	4.13051	0.16040	1651.31	10641	
35	4.11295	0.17989	1644.29	10664	
36	3.87631	0.20966	1549.68	10974	
37	3.86334	0.33502	1544.49	10991	
38	3.85647	0.49772	1541.75	11000	
39	3.85265	0.38111	1540.22	11005	
40	3.84425	0.63931	1536.87	11016	
41	3.83662	0.39411	1533.81	11026	
42	3.83280	0.51341	1532.29	11031	
43	3.82517	0.36634	1529.24	11041	
44	3.81296	0.23818	1524.35	11057	
45	3.76334	0.10740	1504.52	11122	
46	3.75723	0.10787	1502.08	11130	
47	3.74654	0.24683	1497.80	11144	
48	3.74425	0.15079	1496.89	11147	
49	3.73891	0.18680	1494.75	11154	
50	3.72975	0.13429	1491.09	11166	
51	3.72593	0.16958	1489.56	11171	
52	3.72135	0.16516	1487.73	11177	
53	3.70838	0.15702	1482.56	11194	
54	3.70532	0.20963	1481.32	11198	
55	3.69922	0.21425	1398.93	11468	
56	3.48777	0.15752	1394.35	11483	
57	3.48548	0.22505	1393.43	11486	12.51
58	3.47097	0.13086	1387.63	11505	
59	2.84502	0.48178	1137.39	12325	
60	2.83516	0.35204	1133.42	12338	
61	2.81372	0.48347	1124.86	12366	
62	2.80609	0.41017	1121.83	12376	13.43
63	2.69183	0.53931	1076.05	12526	
64	2.68166	0.56430	1072.08	12539	
65	2.65800	0.65372	1062.62	12570	7.77
66	2.64808	0.63403	1058.65	12583	
67	2.35571	2.61120	941.77	12966	+ 6
68	2.25877	0.50723	903.02	13093	
69	2.23892	0.54138	895.08	13119	
70	2.22518	0.46140	889.59	13137	
71	2.20536	0.45036	881.65	13163	
72	2.17251	13.00911	868.53	13206	+++ 7.14
73	2.12365	0.13311	849.00	13270	1343
74	2.04579	0.66131	817.87	13372	
75	2.00915	0.82413	803.22	13420	
76	1.98396	0.81436	793.15	13453	
77	1.97403	0.63918	789.18	13466	
78	1.96564	0.72810	785.83	13477	
79	1.93892	0.88387	775.15	13512	
80	1.92976	0.56873	771.48	13524	4
81	1.92060	0.67067	767.82	13536	
82	1.91220	0.65209	764.47	13547	
83	1.90304	0.51956	760.80	13559	
84	1.89388	0.47665	757.14	13571	
85	1.88625	0.22313	754.09	13581	
86	1.87633	0.21354	750.12	13594	
87	1.86869	0.37384	747.07	13604	
88	1.85877	0.31288	743.10	13617	
89	1.85342	0.43330	740.97	13624	
90	1.84884	0.54529	739.14	13630	
91	1.84274	0.35213	736.69	13638	
92	1.83663	0.33970	734.25	13646	
93	1.82823	0.30126	730.90	13657	
94	1.81984	0.36071	727.54	13668	
95	1.79770	0.18736	718.69	13697	
96	1.71907	0.19656	687.26	13800	
97	1.68854	0.97399	675.05	13840	
98	1.67327	0.31122	668.95	13860	
99	1.66259	0.65576	664.67	13874	
100	1.65190	0.67322	660.40	13888	
101	1.55266	24.74699	620.73	14018	+++++
102	1.54961	23.68475	619.51	14022	+++++
103	1.52289	2.25819	608.83	14057	
104	1.51908	2.14206	607.30	14062	
105	1.50915	2.30518	603.33	14075	
106	1.50534	2.03049	601.81	14080	
107	1.49694	1.34147	598.45	14091	
108	1.48778	1.63270	594.79	14103	
109	1.47785	1.78724	590.82	14116	
110	1.46488	2.09681	585.63	14133	
111	1.45572	1.46173	581.97	14145	
112	1.44885	1.68220	579.22	14154	
113	1.44350	2.15006	577.09	14161	
114	1.43816	1.24164	574.95	14168	
115	1.43129	1.03275	572.20	14177	
116	1.42137	0.99580	568.24	14190	
117	1.41297	1.10303	564.88	14201	
118	1.40610	0.79794	562.13	14210	
119	1.39770	0.64852	558.78	14221	
120	1.38549	1.82600	553.89	14237	
121	1.38167	1.78330	552.37	14242	
122	1.37327	2.04219	549.01	14253	
123	1.36037	1.41708	539.86	14283	
124	1.34274	0.83533	536.80	14293	
125	1.32747	1.39920	530.70	14313	
126	1.31984	1.38789	527.65	14323	
127	1.31144	1.51146	524.29	14334	
128	1.29847	1.01536	519.10	14351	
129	1.29312	1.05290	516.97	14358	
130	1.27786	1.50170	510.86	14378	
131	1.27404	1.64288	509.34	14383	
132	1.26259	2.70069	504.76	14398	+
133	1.25419	6.42871	501.40	14409	+
134	1.24503	2.32149	497.74	14421	
135	1.24198	1.52839	496.52	14425	
136	1.23053	1.11573	491.94	14440	
137	1.22747	1.52209	490.72	14444	
138	1.21373	32.96595	485.23	14462	+++++++
139	1.18778	2.79381	474.85	14496	+
140	1.16946	0.62663	467.53	14520	
141	1.16030	0.87387	463.87	14532	
142	1.14274	0.51726	456.85	14555	
143	1.13893	0.53558	455.32	14560	
144	1.13434	0.55867	453.49	14566	
145	1.12442	0.43039	449.52	14579	
146	1.10763	0.40549	442.81	14601	
147	1.09160	7.52555	436.40	14622	++
148	1.07480	7.34448	429.69	14644	+
149	1.05572	0.30532	422.06	14669	
150	1.00915	0.11803	403.44	14730	
151	0.93893	0.11868	375.37	14822	
152	0.93206	0.12279	372.62	14831	6.79
153	0.92061	0.16872	368.04	14846	
154	0.91450	0.15377	365.60	14854	
155	0.89618	0.45087	358.28	14878	
156	0.89236	0.36908	356.75	14883	
157	0.88015	1.10219	351.87	14899	
158	0.86183	0.92495	344.54	14923	
159	0.85572	5.69947	342.10	14931	+
160	0.83893	5.84266	335.39	14953	+ 6.91
161	0.69923	0.21725	279.54	15136	
162	0.66488	0.10733	265.81	15181	
163	0.62442	0.10717	249.63	15234	
164	0.53282	11.16377	213.01	15354	++
165	0.18321	0.88836	73.24	15812	
166	0.14580	0.29633	58.29	15861	
167	0.06870	0.21470	27.47	15962	
168	0.00763	3.17474	3.05	16042	+
169	0.00000	83.23671	0.00	16052	++++++++++++++++++
170	-0.00840	3.40815	-3.36	16063	+
171	-0.03893	0.15635	-15.56	16103	
172	-0.04427	0.14362	-17.70	16110	

10: 58: 02



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OBSET	124.00 kHz
OBFIN	10905.1 Hz
PW1	5.9 us
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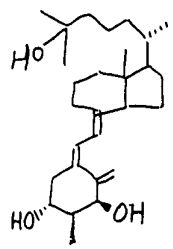
SAMPO	32768
SCANS	9216
DUMMY	0
FREQU	5000.0 Hz
FILTR	5000 Hz
ACQTM	3.277 sec
PD	5.000 sec
RGAIN	24
BF	0.10 Hz
T1	0.0 %
T2	0.0 %
T3	90.0 %
T4	100.0 %
EXMOD	SGNON
DFILE	[100, 140]FN0344
SHMFL	TH5
SPEED	15 Hz
OPERATOR	J.SHIMODE



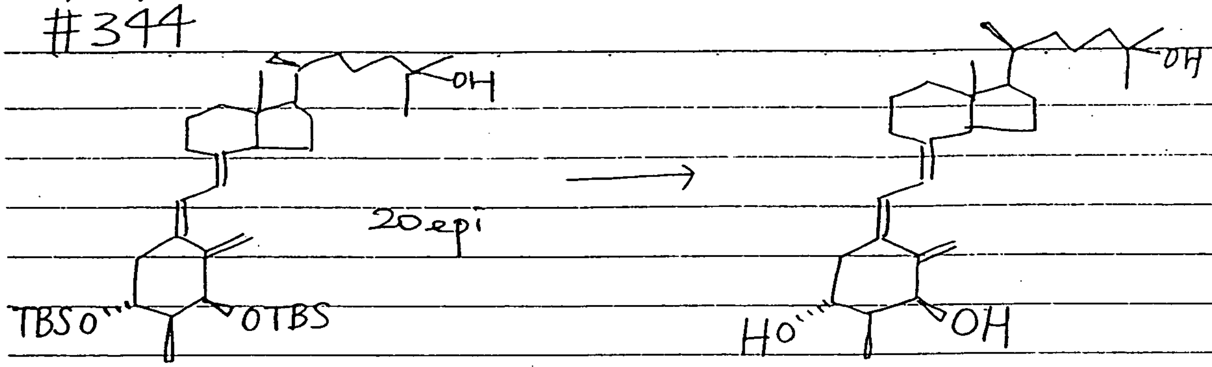
Experimental note of compound synthesis with English translation

Compound (72) / 20epi Ds / # 344

33.8904
9226

33.5847
594
1.0 mg

#344



MW MW 430.67

[protective material]

#343 [ホゴ体] work up
CSA MW 232.30 11mg [stirring under Ar at rt]
MeOH 1 ml
[Ar下 rtかくはん] 14:20〜 ~9:00
250ml

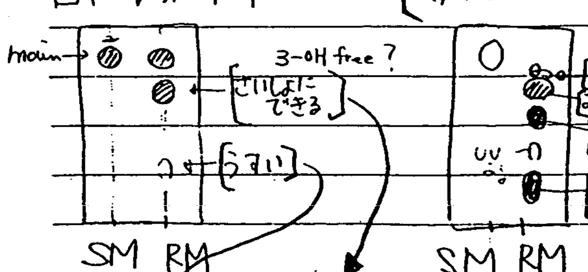
[MeOHを留去し. 水を加え EA抽出. brine洗い.
MgSO4上 脱水. ろか. エバポレート.
シリカゲルカラム (φ0.9 cm 10cm height, EA:n-hex = 1:1)
にて精製]

9.3mg (y. 63%) →HPLCで分離.

MeOH was distilled away, water was added, extracted with EA, washed with
dried over MgSO4, dehydration, filtration, evaporation
purification by silica-gel column chromatography
(φ 0.9 cm 10 cm height, EA/n-Hex = 1:1)

→ Separation by HP

EA:n-hex = 1:1



[red]
[purple no UV]
[this spot has UV blue → one remove
[this spot has UV blue → two remove
[appeared first]
[light]

KOKUYO NR SERIES

Exhibit 1
Note 2
(Translation)

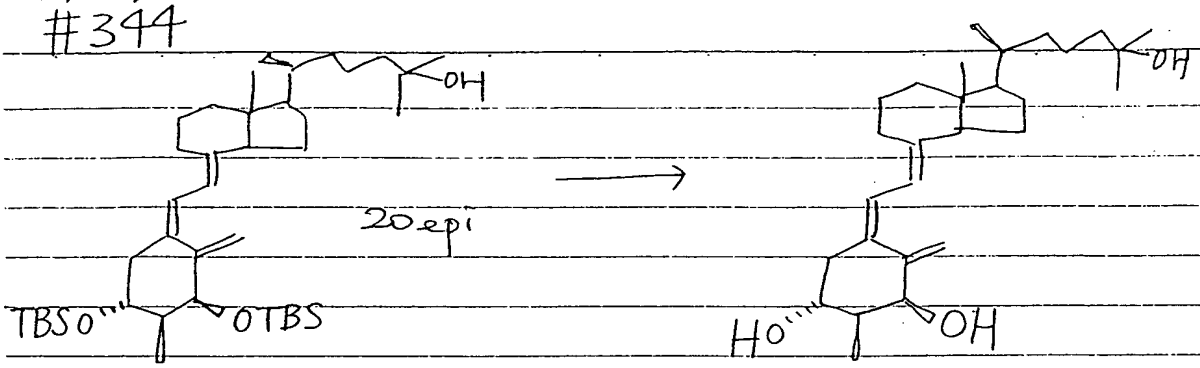
33.8904
9:05

30.2

33.5847
5:54

1.0 mg

#344



MW

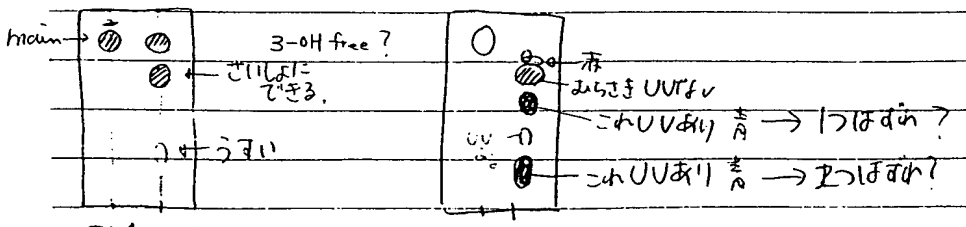
MW 430.67

#343 ホゴ体 work up
CSA MW 232.30 11mg
MeOH 1ml
Ar下 セプタムにて 14:20～ 9:00
～50ml

MeOHを留去し、水を加え EA抽出、brineで洗い、
$MgSO_4$上 脱水、ろ過 エバポレート、
シリカゲルカラム（φ0.9cm 10cm height, EA=n-hex=1:1）
にて精製

9.3mg (y. 63%) → HPLCで1分離

EA:n-hex=1:1



KOKUYO MR SERIES

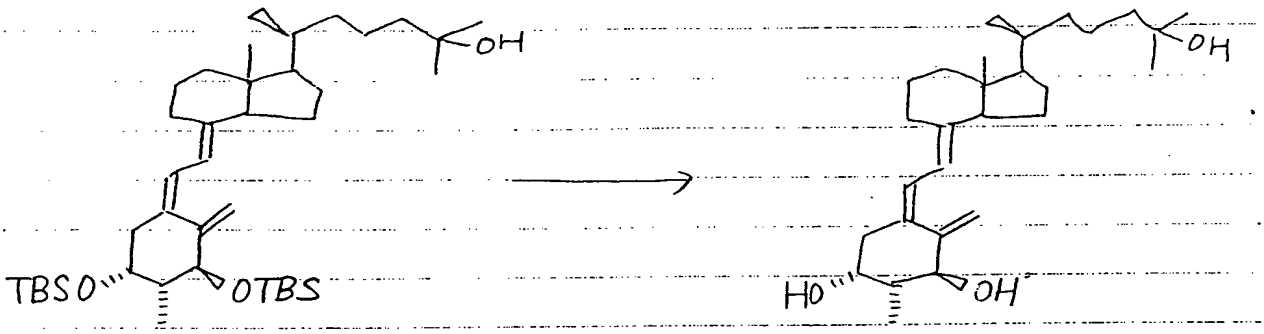
Exhibit 1
Note 2

Experimental note of compound synthesis with English translation

Compound (68) / 20epi Aa / # 346

4.5

#346



MW 659.20 MW

{ #345のworkup
CSA 11mg
MeOH 1mL 20:30~

[rtかくはん後 反応液からMeOHをとばし、水を加えEA抽出.
brine洗い MgSO4上 脱水
ろか、エバポ。] 11:00

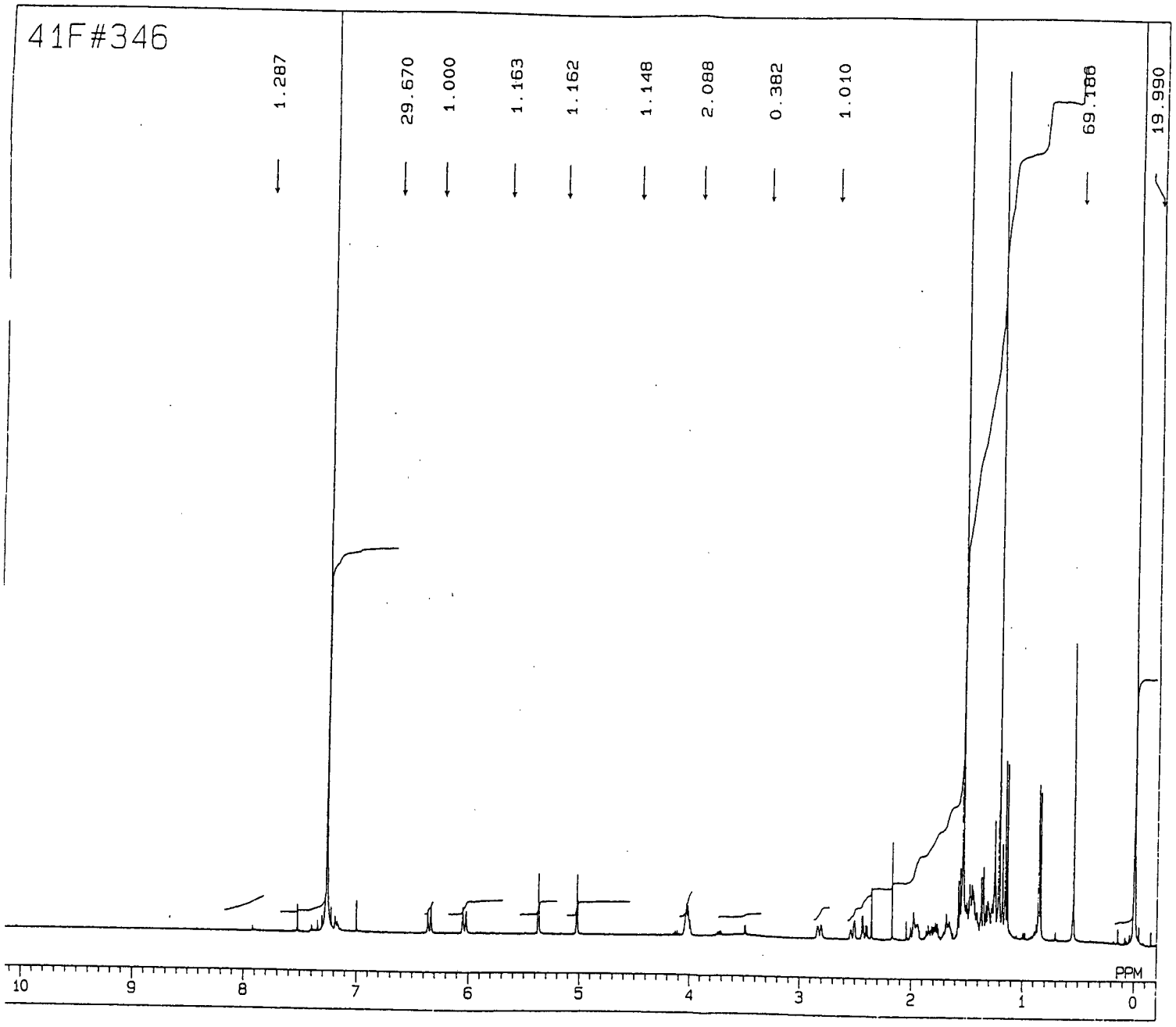
[シリカゲルカラムで分離後 ~~eluted~~
HPLCカラム(ODS(18))で分離。
RP-18] 4.5mg (y.31%)

After stirring at rt, MeOH was evaporated from reaction mixture,
water was added and extracted with EA
washed with brine, dried over MgSO4
filtered, evaporated

After separation by silica gel column chromatography
separation by HPLC column (ODS (18))

Exhibit 1
Chart 1, p. 1

11: 26: 05



SLVNT	CDCL3
OBNUC	1H
OBFRQ	399.65 MHz
OBSET	124.00 kHz
OBFIN	10905.1 Hz
PW1	5.9 us
POINT	32768
SAMPO	32768
SCANS	9216
DUMMY	0
FREQU	5000.0 Hz
FILTR	5000 Hz
ACQTM	3.277 sec
PD	5.000 sec
RGAIN	25
BF	0.10 Hz
T1	0.0 %
T2	0.0 %
T3	90.0 %
T4	100.0 %
EXMOD	SGNON
DFILE	[100, 140]FN0346
SHMFL	TH5
SPEED	15 Hz
OPERATOR	J.SHIMODE

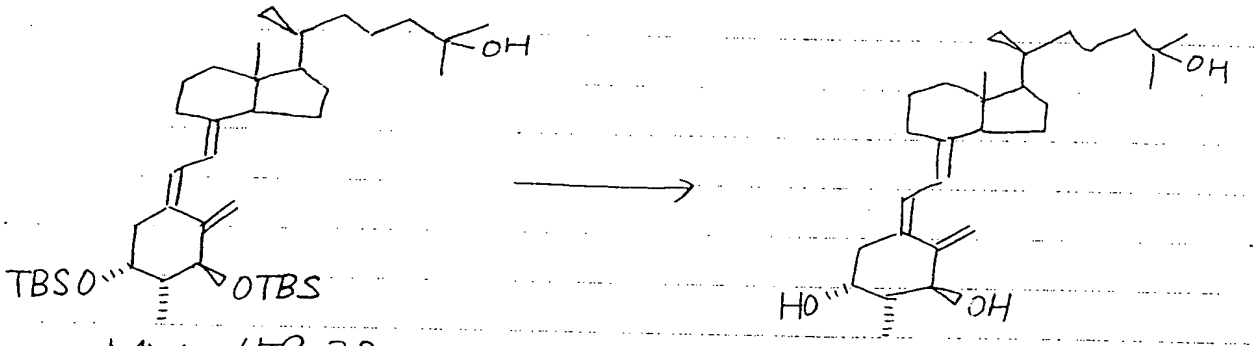
MXINT	686368300
RESOL	0.3100000 Hz
RESOL	0.0007754 ppm
EXREF	0.0000000 ppm
OBS	-2398.38 Hz
ABOBS	399784.9000000 KHz
NGAIN	23
COMNT	41F#346

NO.	PPM	INT(%)	FREQ(Hz)	POSITION	BAR-GRAPH
1	7.91370	0.09737	3163.76	5684	
2	7.51981	0.48494	3006.29	6200	
3	7.40225	0.05175	2959.29	6354	
4	7.39385	0.12111	2955.93	6365	
5	7.34194	0.20745	2935.18	6433	
6	7.30149	0.29605	2919.01	6486	
7	7.26027	100.00000	2902.53	6540	**********
8	7.23813	0.38301	2893.68	6569	
9	7.22057	0.43407	2886.66	6592	
10	7.18469	0.28503	2872.31	6639	
11	7.17859	0.19619	2869.87	6647	
12	7.16837	0.18829	2864.99	6663	
13	7.15798	0.13951	2861.63	6674	
14	7.13889	0.06443	2854.00	6699	
15	6.99614	0.56372	2796.94	6886	
16	6.35951	0.41519	2542.42	7720	
17	6.33126	0.49927	2531.13	7757	
18	6.04730	0.46505	2417.60	8129	
19	6.01905	0.39537	2406.31	8166	
20	5.37402	0.55632	2148.44	9011	
21	5.36944	1.06697	2146.61	9017	
22	5.36486	0.63816	2144.78	9023	
23	5.02593	0.59430	2009.28	9467	
24	5.02135	1.05666	2007.45	9473	
25	5.01753	0.78797	2005.92	9478	
26	4.13128	0.08595	1651.61	10639	
27	4.11295	0.09201	1644.29	10663	
28	4.05189	0.12551	1619.87	10743	
29	4.04349	0.23001	1616.52	10754	
30	4.03128	0.58951	1611.63	10770	
31	4.01601	0.54112	1605.53	10790	
32	4.00837	0.43787	1602.48	10800	
33	3.99311	0.28651	1596.37	10820	
34	3.73967	0.09817	1495.06	11152	
35	3.72670	0.10343	1489.87	11169	
36	3.72212	0.10775	1488.04	11175	
37	3.70914	0.09923	1482.85	11192	
38	3.69156	0.05572	1475.83	11215	
39	3.49922	0.20733	1398.93	11467	
40	3.48548	0.21637	1393.43	11485	
41	2.84121	0.23545	1135.86	12329	
42	2.83128	0.22394	1131.90	12342	
43	2.81148	0.25305	1123.96	12368	
44	2.80098	0.24576	1119.69	12382	
45	2.54044	0.18161	1015.63	12723	
46	2.50752	0.34701	1002.50	12766	
47	2.44197	0.42023	976.26	12852	
48	2.42699	0.43008	971.07	12869	
49	2.40686	0.25118	962.22	12898	
50	2.39464	0.25007	957.34	12914	
51	2.35571	0.91869	941.77	12965	
52	2.17251	1.70768	868.53	13205	
53	2.04579	0.34157	817.87	13371	
54	2.03510	0.05856	813.60	13385	
55	2.00839	0.23253	802.92	13420	
56	1.98167	0.50340	792.24	13455	
57	1.95724	0.28313	782.47	13487	
58	1.94350	0.29859	776.98	13505	
59	1.88549	0.05986	753.78	13581	
60	1.86640	0.18540	746.15	13606	
61	1.85724	0.13716	742.49	13618	
62	1.84732	0.27343	738.53	13631	
63	1.83968	0.16681	735.47	13641	
64	1.82594	0.18811	729.98	13659	
65	1.81755	0.25049	726.62	13670	
66	1.80762	0.22568	722.66	13683	
67	1.80075	0.24723	719.91	13692	
68	1.79465	0.20117	717.47	13700	
69	1.79083	0.24881	715.94	13705	
70	1.78396	0.32445	713.20	13714	
71	1.77785	0.23647	710.75	13722	
72	1.76793	0.29629	706.79	13735	
73	1.76106	0.22537	704.04	13744	
74	1.74350	0.07263	697.02	13767	
75	1.71144	0.15742	684.20	13809	
76	1.68549	0.47277	673.83	13843	
77	1.67404	0.29028	669.25	13858	
78	1.65953	0.34395	663.45	13877	
79	1.62442	0.13878	649.41	13923	
80	1.59770	0.10967	638.73	13958	
81	1.57938	1.06582	631.41	13982	
82	1.56411	1.27070	625.31	14002	
83	1.54198	35.76949	616.46	14031	**********
84	1.51526	0.63027	605.77	14066	
85	1.50305	0.67486	600.89	14082	
86	1.48091	1.00631	592.04	14111	
87	1.47098	0.78546	588.07	14124	
88	1.45877	0.98550	583.19	14140	
89	1.45114	0.91351	580.14	14150	
90	1.44579	0.88146	578.00	14157	
91	1.43892	0.74082	575.26	14166	
92	1.42289	0.46625	568.85	14187	
93	1.41526	0.50507	565.80	14197	
94	1.40839	0.35961	563.05	14206	
95	1.39923	0.28839	559.39	14218	
96	1.37327	1.13146	549.01	14252	
97	1.35724	1.29766	542.60	14273	
98	1.33205	0.63017	532.53	14306	
99	1.31602	0.70781	526.12	14327	
100	1.29694	0.48567	518.49	14352	
101	1.28396	0.58365	513.31	14369	
102	1.27786	0.58894	510.86	14377	
103	1.27404	0.66511	509.34	14382	
104	1.26259	0.88538	504.76	14397	
105	1.25954	1.14819	503.54	14401	
106	1.25343	2.09371	501.10	14409	
107	1.24579	0.97872	498.05	14419	
108	1.23358	0.43210	493.16	14435	
109	1.22747	0.59758	490.72	14443	
110	1.21526	14.77704	485.84	14459	***
111	1.18320	1.68905	473.02	14501	
112	1.15725	3.12907	462.65	14535	*
113	1.14045	3.06572	455.93	14557	*
114	1.11602	0.19907	446.17	14589	
115	1.07633	0.09577	430.30	14641	
116	1.05648	0.11334	422.36	14667	
117	1.02213	0.06880	408.63	14712	
118	1.01297	0.07266	404.97	14724	
119	0.99694	0.16019	398.56	14745	
120	0.98015	0.14783	391.85	14767	
121	0.93969	0.07757	375.67	14820	
122	0.92061	0.10616	368.04	14845	
123	0.91145	0.09963	364.38	14857	
124	0.89618	0.18550	358.28	14877	
125	0.88016	0.31971	351.87	14898	
126	0.85801	2.73087	343.02	14927	*
127	0.84199	2.58526	336.61	14948	*
128	0.70687	0.16637	282.59	15125	
129	0.54656	5.15249	218.51	15335	*
130	0.50763	0.05559	202.94	15386	
131	0.14580	0.23644	58.29	15830	
132	0.08168	0.09306	32.65	15944	
133	0.04122	0.14523	16.48	15997	
134	0.00763	1.92187	3.05	16041	
135	0.00000	67.77723	0.00	16051	**********
136	-0.00840	2.61810	-3.36	16062	*
137	-0.04046	0.28143	-16.17	16104	
138	-0.14427	-0.05415	-57.68	16240	
139	-0.14962	0.19508	-59.81	16247	

Exhibit 1
Chart 1, p. 2

4.5

#346



MW 659.20

MW

#345のwork up
CSA 11mg
MeOH 1mL 20:30～

rtかくはん後 反応液からMeOHを
とばし、水をかえ EA抽出. 11:00
brine洗い、MgSO4上 脱水
ろか、エバポ.

シリカゲルカラムで分離後 ~~ethyl~~ 4.5mg
HPLCカラム(ODS #18) で分離. (y. 31%)
RP-18

Exhibit 1
Note 1

Experimental note of VDR binding affinity with English translation
Compound (68) / 20epi Aa / # 346 and Compound (72) / 20epi Ds / # 344

Experiment of Bovine Thymus VDR binding affinity (# 7)

① Make phosphate-potassium buffer Keeping at 4℃

② Diluted solution series of 1α,25(OH)2D3, #344, #346

③ Concentration preparation of [26,27-methyl3H] 1α,25(OH)2D3 solution
Take 100 μL and evaporate Add 6.25 mL of Japanese pharmacopeia grade ethanol

④ Pour sample / 50 μL Japanese pharmacopeia grade ethanol (②) into disposable culture tube (12 x 75 mm IWAKI) in concentration order (from thin to dense)
(like ⑭ ㉘ →①⑮)
⑧⑤ → ⑨⑥are Japanese pharmacopeia grade ethanol only (by dispenser)

⑤ Make receptor solution (lot 110431 YAMASA)
Pour 5 mL of phosphate-potassium buffer (①) into a vessel containing thymus receptor and dissolve the receptor gently. Add further 50 mL of the buffer and stir gently

⑥ Add 500 μL of the receptor solution to each tubes except blank (⑧⑨ ⑨⓪ ⑨① ⑨②)
Add 500 μL of the buffer solution to each blank tube

⑦ Stir by vortex, avoid forming

⑧ Pre incubate at rt for 1 hr
Put the top on the tubes by plastic wrap & aluminum foil
13:40 ～ 14:40 rt approximately 22℃

RI room

⑨ Add 50 μL of the hot solution (③) to each tubes by dispenser
In case of hot only count (⑨⑦ ⑨⑧ ⑨⑨ ⑩⑩), hot solution is added to vial tube

⑩ Stir by vortex, avoid forming

⑪ Put the top on the tubes by plastic wrap, put the tubes into 4℃ refrigerator in RI room, and stand overnight 15:10～

97	16217.7 dpm	
98	16349.9	
99	16280.0	
100	16634.8	
101	54.3	
102	28.3	
103	42.7	
104	56.9	Average 16370 dpm
		" 45 dpm

Add 10 mL of ACS-II and measure radioactivity count for 1 min by Aloka A
Stand rt and measure radioactivity count for 2 min tomorrow

(16370 dpm = 273 dps = 273 Bq
11.4 GBq / mg therefore 24 pg / tube)

～9:25

⑫ Put out the yesterday's samples from the refrigerator in RI room and add 200 μL of DCC solution (lot M602 YAMASA) to each tubes by dispenser except total count tubes (⑨③ ⑨④ ⑨⑤ ⑨⑥)

Add the buffer solution ① to each total count tubes

⑬ Vortex tubes

⑭ Stand for 30 min at 4℃ 9:50～10:20

10:30～10:40

⑮ Centrifuge at 3000 rpm for 10 min at 0℃

⑯ Transfer 500 μL of supernatant to 20 mL WHEATON vial

Lay ice on tray and put tube on the ice

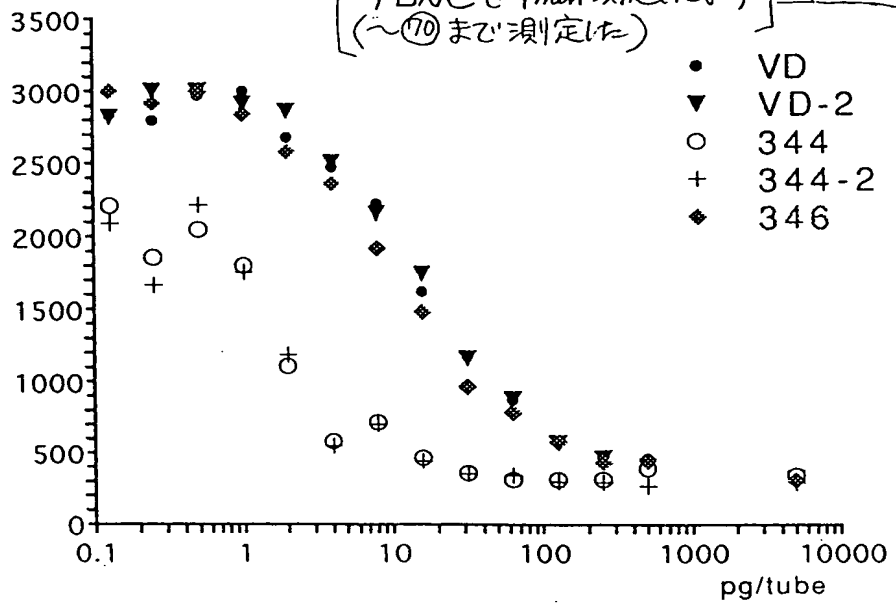
in concentration order (from thin to dense) ①→⑭ same pipetter tip

Change pipetter tip ⑮→㉘

⑰ Add 9.5 mL of ACS-II to each tubes, shake, and measure radioactivity count (2 min) Aloka A

☆ ハッリア
☆ バイアル
☆ 1分注器
☆ ピペットマン1000
200
☆ "

☆ レレーバ
☆ チップたくさん



This shows the results of 1 min measuring by Aloka C
(measured to ~ (70))

MR

L3 オレンジ みどり

/50μl	1α25(OH)$_2$VD3		#344		#346	
5ng	290	325	308	296	338	305
500pg	357	363	325	312	445	386
250	444	529	318	302	445	477
125	608	623	326	324	528	573
63	802	806	349	326	698	623
32	1094	1166	391	387	1041	913
16	1701	1676	458	369	1395	1357
8	2164	2109	658	663	1834	1822
4	2494	2511	568	520	2428	2180
2	2519	2536	1145	1161	2766	2499
1	2879	2768	1739	1819	2768	2763
0.5	2862	2924	2081	2062	2762	2768
0.25	2851	2959	1942	1847	2910	2834
0.13	2839	2690	1987	1932	2990	2694

0	85 2744	86 2982	87 3149	88 3048	2980
blank	89 224	90 166	91 174	92 311	218
total count	93 7965	94 8280	95 8052	96 8325	8155
[入れた量]	97 16184	98 15926	99 16360	100 16561	16257
blank	101 27	102 59	103 43	104 34	40

{ added amount }

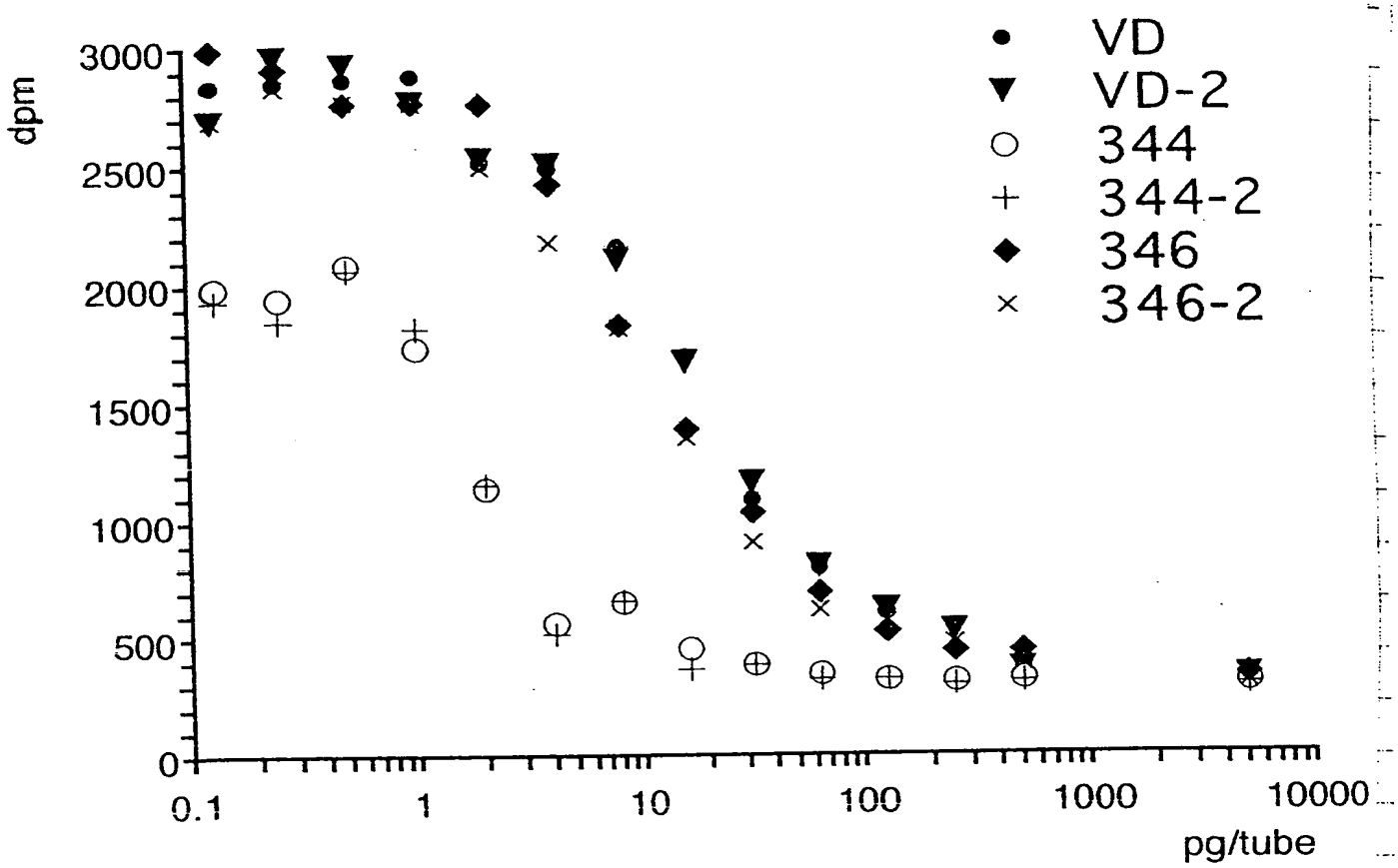
(2762)

すべての実験値から218をひいて (2980−218)で
割り ×100して Bound[%]をだします

{ Bound[%] was calculated as follows: Subtract 218 from all experimental values, then this value divides by (2980 - 218) and multiply 100. }

50
50+500+200

#B7



	pg/tube	VD	VD-2	344	344-2	346	346-2
0	5000.0	290.00	325.00	308.000	296.00	338.00	305.00
1	500.00	357.00	363.00	325.000	312.00	445.00	386.00
2	250.00	444.00	529.00	318.000	302.00	445.00	477.00
3	125.00	608.00	623.00	326.000	324.00	528.00	573.00
4	63.000	802.00	806.00	349.000	326.00	698.00	623.00
5	32.000	1094.0	1166.0	391.000	387.00	1041.0	913.00
6	16.000	1701.0	1676.0	458.000	369.00	1395.0	1357.0
7	8.0000	2164.0	2109.0	658.000	663.00	1834.0	1822.0
8	4.0000	2494.0	2511.0	568.000	520.00	2428.0	2180.0
9	2.0000	2519.0	2536.0	1145.00	1161.0	2766.0	2499.0
10	1.0000	2879.0	2768.0	1739.00	1819.0	2768.0	2763.0
11	0.50000	2862.0	2924.0	2081.00	2062.0	2762.0	2768.0
12	0.25000	2851.0	2959.0	1942.00	1847.0	2910.0	2834.0
13	0.13000	2839.0	2690.0	1987.00	1932.0	2990.0	2694.0

dpm

<Results>

blank = 224 + 166 + 174 + 311 / 4 = 218

0 = 2744 + 2982 + 3149 + 3048 / 4 = 2980

Bound[%] was calculated as follows: Subtract 218 which is average value of blank from all experimental values, then this value divides by (subtract 218 from 2980 which is average value of drug 0)(2980 – 218 = 2762) and multiply 100

total count = 7965 + 8280 + 8052 + 8325 / 4 = 8155 dpm

8155 / 60 dps = 136 Bq As I put 500 μL from 800 μL and measured radioactivity count

136 x 8 / 5 = 217 Bq

11.4 GBq / mg therefore 19 pg / tube J

As average added amount is 16257 dpm

from 271 Bq

24 pg / tube J

Approximately 80% of hot receptor exists in solution

and the rest should absorb an inside wall of glass tube

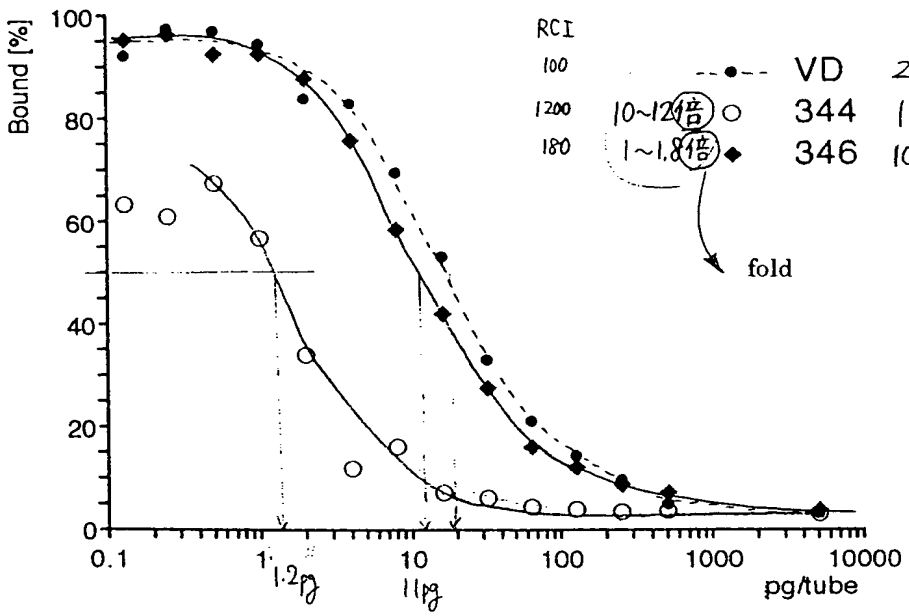
217 Bq / tube = 217 / 4.85T / (50 + 500 + 50) μL

= 0.075 nM

Or, it may exists as 1α25(OH)2 and the rest may count of decompose stuff

Bovine
Chicken

#B7(edit)



pg/tube	VD	VD-2	VD-	344	344-2	344-	346	346-2	346-
5000.0	2.6068	3.8740	3.2404	3.25851	2.8240	3.0413	4.3447	3.1499	3.7473
500.00	5.0326	5.2498	5.1412	3.87400	3.4033	3.6387	8.2187	6.0825	7.1506
250.00	8.1825	11.260	9.7212	3.62056	3.0413	3.3309	8.2187	9.3773	8.7980
125.00	14.120	14.663	14.392	3.91021	3.8378	3.8740	11.224	12.853	12.038
63.000	21.144	21.289	21.217	4.74294	3.9102	4.3266	17.379	14.663	16.021
32.000	31.716	34.323	33.020	6.26358	6.1188	6.1912	29.797	25.163	27.480
16.000	53.693	52.788	53.240	8.68936	5.4671	7.0782	42.614	41.238	41.926
8.0000	70.456	68.465	69.461	15.9305	16.112	16.021	58.508	58.074	58.291
4.0000	82.404	83.020	82.712	12.6720	10.934	11.803	80.014	71.035	75.525
2.0000	83.309	83.925	83.617	33.5626	34.142	33.852	92.252	82.585	87.419
1.0000	96.343	92.324	94.334	55.0688	57.965	56.517	92.324	92.143	92.234
0.50000	95.728	97.972	96.850	67.4511	66.763	67.107	92.107	92.324	92.216
0.25000	95.329	99.240	97.285	62.4185	58.979	60.699	97.466	94.714	96.090
0.13000	94.895	89.500	92.198	64.0478	62.056	63.052	100.36	89.645	95.004

Bovine Thymus VDRへの結合実験（#7）

① リン酸カリバッファを作製　4℃保存
② 1α25(OH)2VD3, #344, #346の希釈系列
③ [26,27-methyl 3H] 1α,25(OH)2VD3の濃度調整
100μlとってとばし　6.25mlの局エタ

④ disposable culture tube (12×75mm イワキ)に sample / 50μl局エタ(②)をうすい順に入れていく
（⑭⑮ → ①⑮のように）
⑧⑨ → ⑯は局エタのみ.（分注器で）

⑤ レセプタ溶液をつくる（lot 11043 ヤマサ）
Thymus Receptorの容器にリン酸カリバッファ①を5ml加えて静かにとかす. さらに50mlを加え静かにまぜる.

⑥ レセプタ溶液500μlを blank (⑧⑨⑩⑪⑫) 以外のtubeに加える.
加えなかったtubeには bufferを500μl加える

⑦ vortexであわだてないようにかくはんする

⑧ rtで1hr pre incubation
ラップ & ホイルでふた　rt 22℃ぐらい
13:40 ～ 14:40

#

RI室

⑨ hot溶液(③)をすべてのtubeに分注器で50μlずつ加える.
hotのみcount(⑨⑦ ⑨⑧ ⑨⑨ ⑩⑩)にはバイアルに入れる.

⑩ vortexであわだてないようにかくはんする

⑪ ラップでふたをして 4℃のRI室の冷蔵庫に入れ over night. 15:10〜

97	16217.7 dpm
98	16349.9
99	16280.0
100	16634.8
101	54.3
102	28.3
103	42.7
104	56.9

平均 16370 dpm
45 dpm

10mlのACS-IIを加えてアロカAで1min countする.
RTで放置し次の日にいっしょに2min count.

(16370 dpm = 273 dps = 273 Bq
11.4GBq/mg だから 24pg/tube)

遠心 0℃ スイッチon

～9:25 RI室の

⑫ 前日のサンプルを冷蔵庫から出して total count (㊸㊹㊺㊻)以外の tubeに DCC液を (lot M602 ヤマサ) 200μlずつ 分注器でかえる かえなかった tubeには ①のバッファをかえる

⑬ tubeを vortex

⑭ 4℃で 30min 放置 9:50～10:20

10:30～10:40

⑮ 遠心 0℃ 10min 3000rpm

⑯ 上澄を 500μlずつ WHEATONの 20mlの バイアルに 移す バットの上に引くをしいてしけんかん たてをならべる

(うすい順に ①→⑭ チップ同じ
チップかえて ⑮→㉘

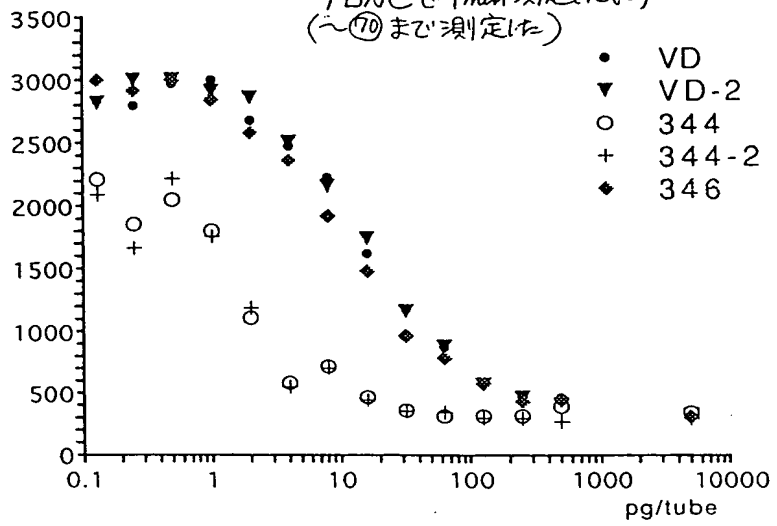
⑰ ACS-Ⅱ を 9.5mlずつ加えて shakeし count (2min) する アロカA

☆ バッファ
☆ バイアル
☆ ノ分注器
☆ ピペットマン1000
200
☆

☆ レベル
☆ チップたくさん.

Data #B7

アロカCで1min測定したもの
(～⑦⓪まで測定した)



L3 オレンジ みどり

/50µl	1α,25(OH)$_2$VD3		#344		#346	
5ng	1 290	15 325	29 308	43 296	57 338	71 305
500pg	2 357	16 363	30 325	44 312	58 445	72 386
250	3 444	17 529	31 318	45 302	59 445	73 477
125	4 608	18 623	32 326	46 324	60 528	74 573
63	5 802	19 806	33 349	47 326	61 698	75 623
32	6 1094	20 1166	34 391	48 387	62 1041	76 913
16	7 1701	21 1676	35 458	49 369	63 1395	77 1357
8	8 2164	22 2109	36 658	50 663	64 1834	78 1822
4	9 2494	23 2511	37 568	51 520	65 2428	79 2180
2	10 2519	24 2536	38 1145	52 1161	66 2766	80 2499
1	11 2879	25 2768	39 1739	53 1819	67 2768	81 2763
0.5	12 2862	26 2924	40 2081	54 2062	68 2762	82 2768
0.25	13 2851	27 2959	41 1942	55 1847	69 2910	83 2834
0.13	14 2839	28 2690	42 1987	56 1932	70 2990	84 2694

Exhibit 1
Note 3, p. 5

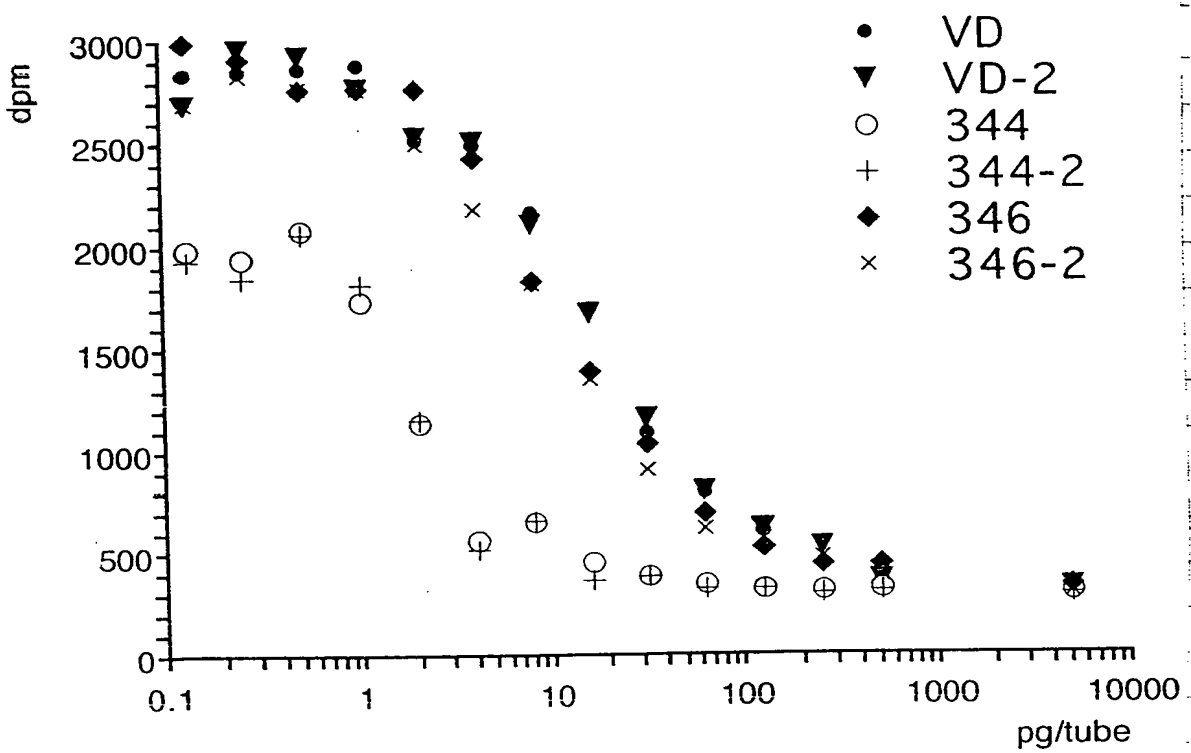
0	85 2744	86 2982	87 3149	88 3048	2980
blank	89 224	90 166	91 174	92 311	218
total count	93 7965	94 8280	95 8052	96 8325	8155
入射量	97 16184	98 15926	99 16360	100 16561	16257
blank	101 27	102 59	103 43	104 34	40

(2762)

すべての実験値から 218 をひいて (2980−218) で
割り ×100 して Bound [%] をだしたん

$$\frac{50}{50+500+200}$$

#B7



	pg/tube	VD	VD-2	344	344-2	346	346-2
0	5000.0	290.00	325.00	308.000	296.00	338.00	305.00
1	500.00	357.00	363.00	325.000	312.00	445.00	386.00
2	250.00	444.00	529.00	318.000	302.00	445.00	477.00
3	125.00	608.00	623.00	326.000	324.00	528.00	573.00
4	63.000	802.00	806.00	349.000	326.00	698.00	623.00
5	32.000	1094.0	1166.0	391.000	387.00	1041.0	913.00
6	16.000	1701.0	1676.0	458.000	369.00	1395.0	1357.0
7	8.0000	2164.0	2109.0	658.000	663.00	1834.0	1822.0
8	4.0000	2494.0	2511.0	568.000	520.00	2428.0	2180.0
9	2.0000	2519.0	2536.0	1145.00	1161.0	2766.0	2499.0
10	1.0000	2879.0	2768.0	1739.00	1819.0	2768.0	2763.0
11	0.50000	2862.0	2924.0	2081.00	2062.0	2762.0	2768.0
12	0.25000	2851.0	2959.0	1942.00	1847.0	2910.0	2834.0
13	0.13000	2839.0	2690.0	1987.00	1932.0	2990.0	2694.0

dpm

Exhibit 1
Note 3, p. 7

#B2 88% 9/12 B7 80%
#B3 84% 9/19 9/30
#B4 84% 9/21

〈結果〉

$$blank = \frac{224 + 166 + 174 + 311}{4} = 218$$

$$0 \quad (cold) = \frac{2744 + 2982 + 3149 + 3048}{4} = 2980$$

すべての実験値から blankの平均値 218 をひいて drug 0のときの平均 2980から 218をひいたもの (2980−218 = 2762)で除し 100をかけ 総合率を計算した.

$$total\ count = \frac{7965 + 8280 + 8052 + 8325}{4}$$

$$= 8155\ dpm$$

$8155/60 = 136$ Bq (dps)

800μl中500μlとってcountしたので"

$136 \times \frac{8}{5} = 217$ Bq

11.4GBq/mgなので 19pg/tube

入れた量の平均は 16257 dpm であるので

271 Bq より

24 pg/tube

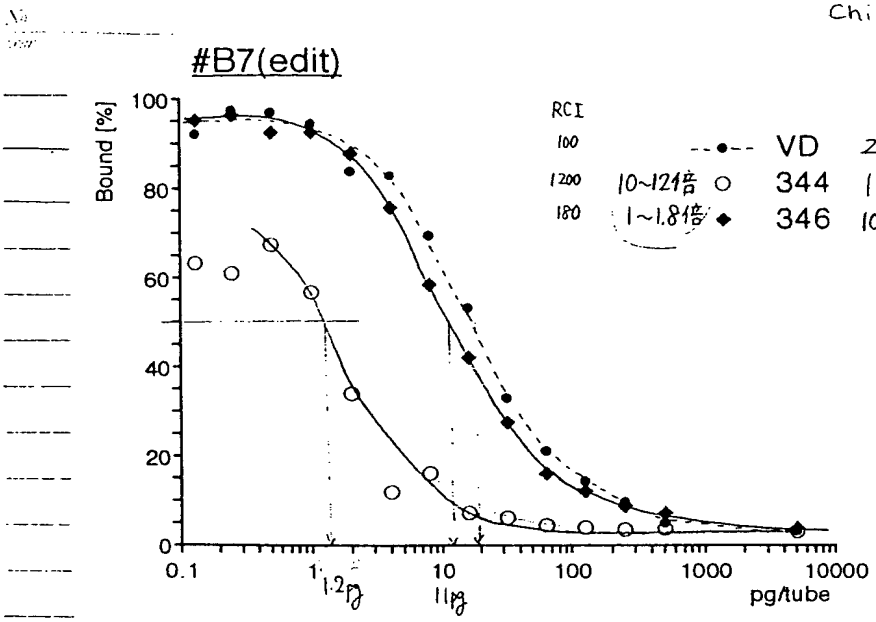
80%ぐらいが溶液中に存在し
あとはガラス壁等に吸着していると考えられる.

$217\ Bq/tube = 217/4.85T/(50+500+50)\mu l$

$= 0.075nM$

又は 1α2分けとしてなお あとにこれたものの countオかもしれない

Bovine
Chicken



pg/tube	VD	VD-2	VD-	344	344-2	344-	346	346-2	346-
5000.0	2.6068	3.8740	3.2404	3.25851	2.8240	3.0413	4.3447	3.1499	3.7473
500.00	5.0326	5.2498	5.1412	3.87400	3.4033	3.6387	8.2187	6.0825	7.1506
250.00	8.1825	11.260	9.7212	3.62056	3.0413	3.3309	8.2187	9.3773	8.7980
125.00	14.120	14.663	14.392	3.91021	3.8378	3.8740	11.224	12.853	12.038
63.000	21.144	21.289	21.217	4.74294	3.9102	4.3266	17.379	14.663	16.021
32.000	31.716	34.323	33.020	6.26358	6.1188	6.1912	29.797	25.163	27.480
16.000	53.693	52.788	53.240	8.68936	5.4671	7.0782	42.614	41.238	41.926
8.0000	70.456	68.465	69.461	15.9305	16.112	16.021	58.508	58.074	58.291
4.0000	82.404	83.020	82.712	12.6720	10.934	11.803	80.014	71.035	75.525
2.0000	83.309	83.925	83.617	33.5626	34.142	33.852	92.252	82.585	87.419
1.0000	96.343	92.324	94.334	55.0688	57.965	56.517	92.324	92.143	92.234
0.50000	95.728	97.972	96.850	67.4511	66.763	67.107	92.107	92.324	92.216
0.25000	95.329	99.240	97.285	62.4185	58.979	60.699	97.466	94.714	96.090
0.13000	94.895	89.500	92.198	64.0478	62.056	63.052	100.36	89.645	95.004

#B7
1 / 1
PO7A
2min

RY NO. 2: [H-3 DPM ESCR 2min 15:07

CYCLE : 1

[1] PRESET TIME (Min.) 2.0
[2] REPEAT 1
[3] CYCLE 1
[4] DATE DPM
[5] ISOTOPE H
[6] B.K.G SUB NO
[7] HEAD PRINT YES

* FUNCTION MODE *

[1] STANDARDIZATION ESCR
[2] CURVE AUTO
[3] REJECT NO
[4] ESCR PRESET TIME (Min.) 0.4
[5] CONSTANT RATIO NO
[6] CLEAR CHECK NO
[7] 2% ERROR NO
[8] FORMATTING NO
[9] FILE NO
[10] REPEAT REPLICATE NO
[11] AWS YES
[12] QUENCHING LEVEL AUTO
[13] BECQUEREL NO
[14] HALF LIFE NO
[15] CALCULATION NO
[16] HISTOGRAM NO

CURVE NO. = 3

H-3 LOW ENERGY Q:N A= -0.00789 B= 0.41072 C= 0.45704 D=-124.77292
H-3 LOW ENERGY Q:H A= 0.00660 B= 0.20210 C= 0.42623 D= -3.11626

NO	ESCR	TIME	H-CPM	H-DPM	H-EFF
1	26.26	2.0	80.5	290.6	27.70
2	26.18	2.0	97.5	357.9	27.24
3	26.20	2.0	121.5	444.1	27.36
4	26.24	2.0	168.0	608.9	27.59
5	26.22	2.0	220.5	802.6	27.47
6	26.20	2.0	299.5	1094.7	27.36
7	26.22	2.0	467.5	1701.8	27.47
8	26.26	2.0	599.5	2164.0	27.70
9	26.20	2.0	682.5	2494.5	27.36
10	26.24	2.0	695.0	2519.1	27.59
11	26.24	2.0	794.5	2879.8	27.59
12	26.26	2.0	793.0	2862.5	27.70
13	26.26	2.0	790.0	2851.6	27.70
14	26.18	2.0	773.5	2839.1	27.24
15	26.22	2.0	89.5	325.8	27.47
16	26.20	2.0	99.5	363.7	27.36
17	26.22	2.0	145.5	529.6	27.47
18	26.20	2.0	170.5	623.2	27.36
19	26.24	2.0	222.5	806.5	27.59
20	26.22	2.0	320.5	1166.5	27.47
21	26.24	2.0	462.5	1676.4	27.59
22	26.22	2.0	579.5	2109.2	27.47
23	26.20	2.0	687.0	2511.0	27.36
24	26.22	2.0	697.0	2536.9	27.47
25	26.22	2.0	760.5	2768.0	27.47
26	26.22	2.0	803.5	2924.5	27.47
27	26.22	2.0	813.0	2959.1	27.47
28	26.28	2.0	748.5	2690.7	27.82
29	26.20	2.0	84.5	308.8	27.36
30	26.22	2.0	89.5	325.8	27.47
31	26.22	2.0	87.5	316.5	27.47
32	26.26	2.0	90.5	326.7	27.70
33	26.24	2.0	96.5	349.8	27.59
34	26.24	2.0	108.0	391.5	27.59
35	26.20	2.0	125.5	458.7	27.36
36	26.26	2.0	182.5	658.8	27.70
37	26.20	2.0	155.5	568.4	27.36
38	26.20	2.0	313.5	1145.8	27.36
39	26.24	2.0	480.0	1739.8	27.59
40	26.22	2.0	572.0	2081.9	27.47
41	26.24	2.0	536.0	1942.8	27.59
42	26.22	2.0	546.0	1987.3	27.47
43	26.20	2.0	81.0	296.1	27.36
44	26.28	2.0	87.0	312.8	27.82
45	26.24	2.0	83.5	302.7	27.59
46	26.24	2.0	89.5	324.4	27.59

Exhibit 1
Chart 3, p. 1

実験セミナー　No. 3　　藤島利江

2-methyl-20epi 1α,25(OH)$_2$VD$_3$誘導体の合成

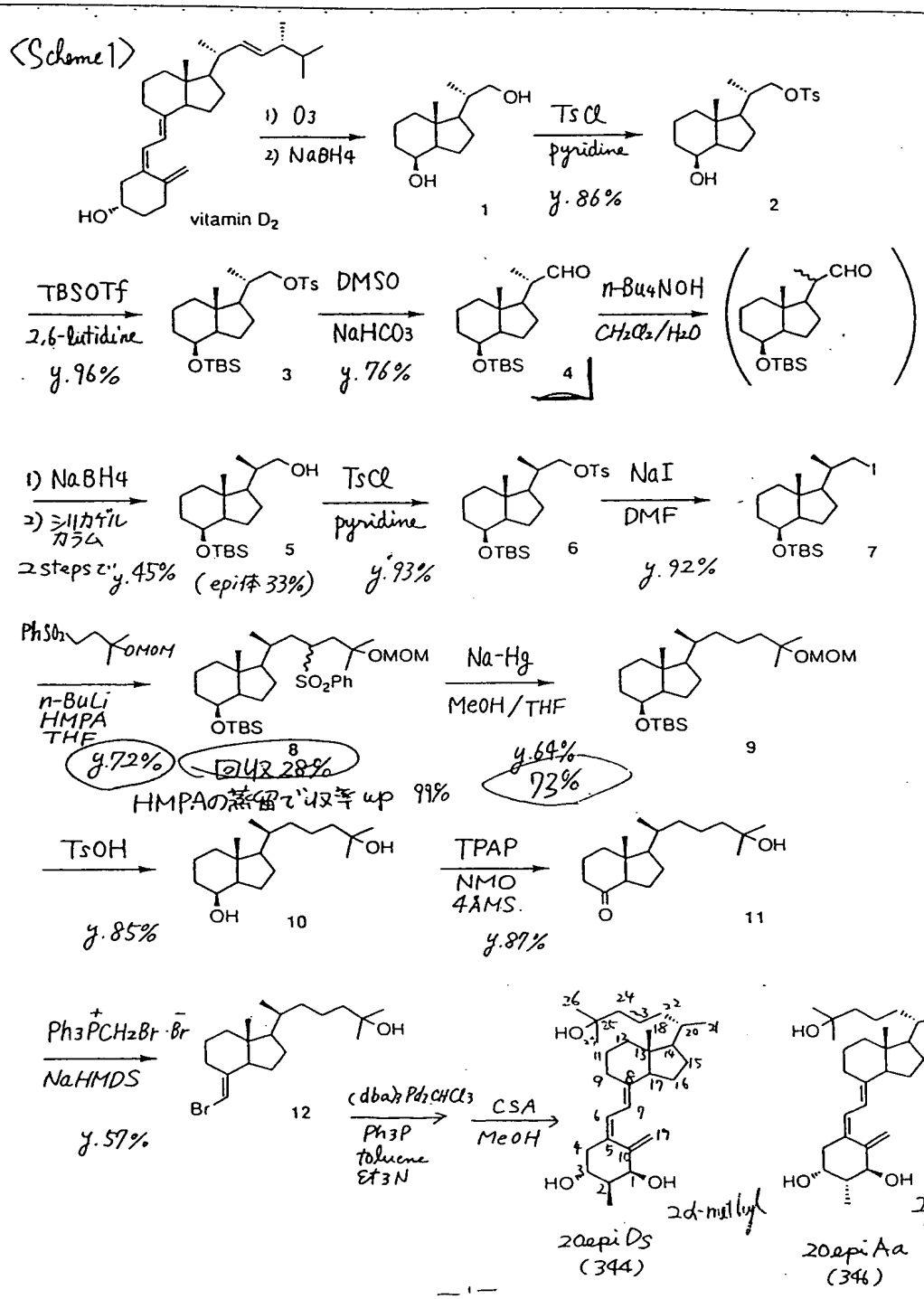
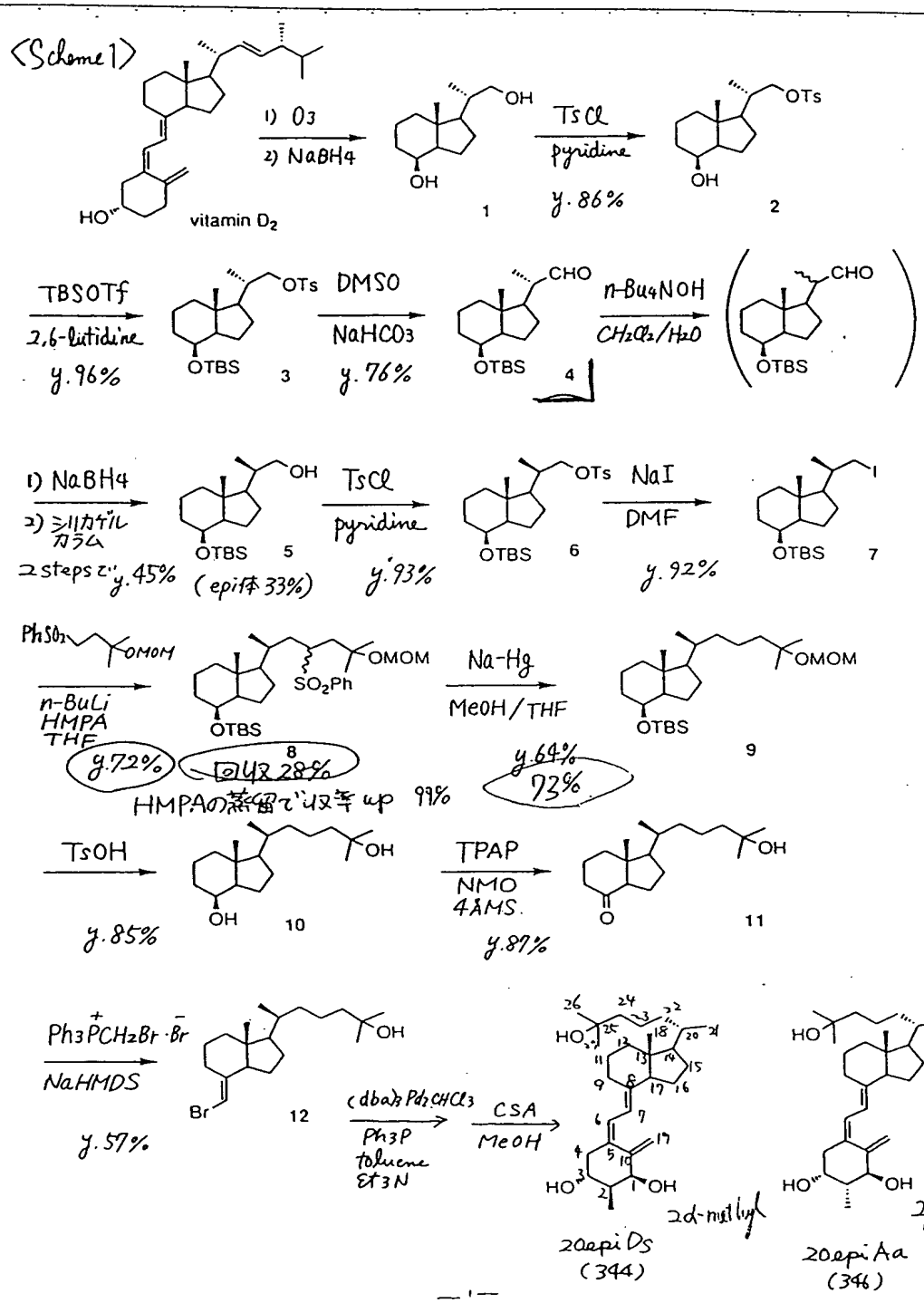


Exhibit 2
p. 1

実験セミナー　No. 3　　藤島利江

◎ 2-methyl-20epi 1α,25(OH)$_2$VD3誘導体の合成



〈Bovine Thymus VDR への結合実験〉

㊗ リン酸カリ buffer
- K_2HPO_4 0.05M
- KH_2PO_4 0.05M
- KCl 0.3M
- DTT 5mM

pH 7.4

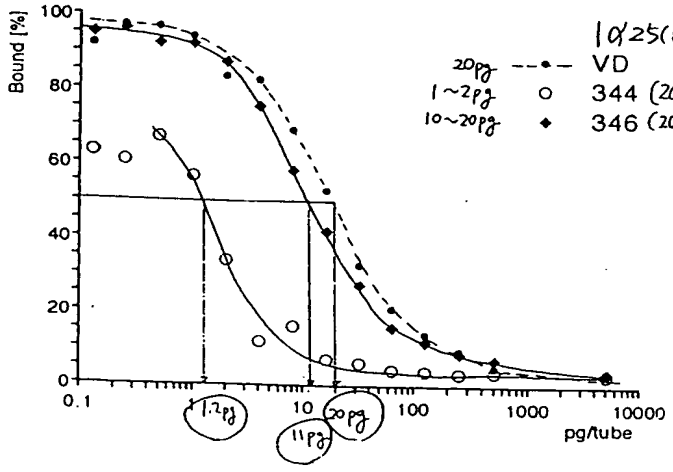
㊗ 1α25(OH)$_2$VD3 #344, #346 と λmax の ε=18000 を用いて濃度調製し希釈系列を作成する.

ウシ胸腺ビタミンＤレセプターはヤマサ醤油株式会社より購入し(lot.110431) 1 アンプル (約 25mg) を 0.05M リン酸 0.5M カリウムバッファー (pH 7.4) 55 ml に溶解した。ビタミンＤ誘導体のエタノール溶液 50 µl とレセプター溶液 500 µl を室温で 1 時間プレインキュベートした後、1α,25-(OH)$_2$[^{3}H]VD$_3$ 溶液 50 µl を最終濃度 0.1nM となるように加えて 4℃で一晩インキュベートした。結合と非結合の 1α,25-(OH)$_2$[^{3}H]VD$_3$ はデキストラン-コーテド-チャコール処理して遠心分離し、上澄に液シンカクテル(ACS-II)を加えて放射活性をカウントした。

ビタミンＤ誘導体の活性は 50%結合阻害する濃度を 1α,25-(OH)$_2$VD$_3$ を 100 としたときの比で表し評価した。

free の drug が DCC にくっついて遠沈する

#B7(edit)



		1α25(OH)2
20pg	VD	100
1~2pg	344 (20epiDs)	1000~1200
10~20pg	346 (20epiAa)	100~180

cf. 20epi 1α,25(OH)$_2$VD3 の VDR への結合活性
- chicken intestine VDR 120
- bovine thymus VDR 500

Exhibit 2
p. 2

Biochemical Pharm
47(6) 987-(19

7 → 8 (#323)
側鎖部 sulfone 980mg (3eq) in dry THF (1.5ml) を Ar 雰囲気下. HMPA 1.5ml (7eq) を加え一様とした後. −78℃に冷却した。 n-BuLi (1.6M in n-hexane) 2.3ml (3eq) を滴下し −78℃で 20min かくはん後 ヨード体 7 525mg (1.20mmol) in dry THF (2+洗い込み 1ml) を滴下. −78℃で 1hr かくはん後 反応液に sat NH4Cl を加えて EA 抽出. 有機層をあわせて brine で洗い. MgSO4 上脱水. ろか. エバポレート. シリカゲルカラム (EA:n-hex=1:8) にて精製し無色 oil 8 503mg (y.72%) を得ると共に 7 145mg の原料を回収 (28%).

8 ^{1}H-NMR (CDCl3/TMS/400MHz) δ −0.02 (3H, s) 0.00 (3H, s) 0.66 (3H, d, J=6.4Hz) 0.85 & 0.88 (3H, s) 1.23 & 1.27 (3H, s) 2.32 (1H, dd, J=15.3Hz, 4.3Hz) 3.26 (1H, m) 3.30 (3H, s) 3.96 (1H, m) 4.57 (1H, d, J=7.3Hz) 4.67 (1H, d, J=7.3Hz) 7.55 (2H, t, J=6.3Hz) 7.63 (1H, t, J=6.3Hz) 7.88 (2H, d, J=6.3Hz)

MS: 580 (M+)

HRMS: calcd for $C_{32}H_{56}O_5SiS$ = 580.3620
found = 580.3618

8 → 9 (#310)
8 165mg (0.28mmol) を dry THF 3ml. dry MeOH 3ml にとかし Na_2HPO_4 3.0g. 5% Na-Hg 9.8g を加えて Ar 下 rt でかくはん overnight. 反応液を ether で希釈し セライトろか. 有機層を brine で洗い. MgSO4 上脱水 ろか. エバポレート. シリカゲルカラム (EA:n-hex=1:9) にて精製 9 無色 oil 80mg (y.64%) を得ると共に原料 11mg (7%) を回収.

9 ^{1}H-NMR (CDCl3/TMS/400MHz) δ −0.01 (3H, s) 0.01 (3H, s) 0.81 (3H, d, J=6.7Hz) 0.89 (9H, s) 0.91 (3H, s) 1.21 (6H, s) 0.98−1.57, 1.64−1.94 (19H, m) 3.36 (3H, s) 3.99 (1H, m) 4.70 (2H, s)

MS: 440 (M^+), 425 $(M-Me)^+$

HRMS: calcd for $C_{26}H_{52}O_3Si$ = 440.3688
found = 440.3687

9 → 10 (#316)
ホゴ体 9 80mg (0.18 mmol) を MeOH 3ml に溶かし. TsOH·H2O 174mg (0.91 mmol) を加えて rt かくはん overnight. 反応液から MeOH を エバポレートし, シリカゲルカラム (EA:nhex = 1:2) にて精製. 無色 oil 43mg (y.85%) を得る.

10 ^{1}H-NMR (CDCl3/TMS/400MHz) δ 0.84 (3H, d, J=6.7Hz) 0.93 (3H,S) 1.21 (6H,S) 4.07 (1H, m)

MS: 264 $(M-H_2O)^+$, 246 $(M-2H_2O)^+$
HRMS: calcd for $C_{18}H_{32}O$: 264.2455 $(M-H_2O)$
found : 264.2453.

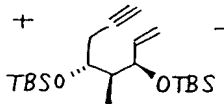
10 → 11 (#326)
アルコール 10 117mg (0.41 mmol) dry CH_2Cl_2 (10ml) 4Å MS 30mg を Ar 下 rt で 5分間かくはんする. TPAP 84mg (0.24 mmol) を加えて 1 hr 20 min 後 反応液を small pad of silica gel 上 ろかし. エバポレート. シリカゲル カラム (EA:nhex = 1:1) にて精製. 100mg (y.87%) を得る.

11 ^{1}H-NMR (CDCl3/TMS/400MHz) δ 0.64 (3H,S) 0.87 (3H, d, J=6.1Hz) 1.22 (6H,S) 2.45 (1H, dd, J=11.6Hz, 7.3Hz)

MS: 262 $(M-H_2O)^+$
HRMS: calcd for $C_{18}H_{30}O$ $(M-H_2O)$: 262.2298
found : 262.2297

11 → 12 (#334)
(bromomethyl)triphenyl phosphonium bromide 389mg (5eq) in dry THF (1.5ml) を Ar 下 −60°C に冷却し 1.0M NaHMDS 0.86ml (4.8eq) を加え −60°C で 1 hr 反応させた後. 11 50mg (0.18 mmol) in dry THF (1.5ml) に transfer する. −60°C → 0°C → rt へと昇温し 1 hr 反応させた. 反応液に n-ヘキサンを加え セライトろかし ろ液を エバポレートして シリカゲルカラム (EA:nhex = 1:8 → 1:3) にて精製. 12 36mg (y.56%) の淡黄 oil を得る.

12 ^{1}H-NMR (CDCl3/TMS/CDCl3) δ 0.56 (3H,S) 0.85 (3H, d, J=6.4Hz) 1.22 (6H,S) 2.88 (1H,m) 5.64 (1H, d, J=1.5Hz)
MS: 356 & 358 (M^+), 338 & 340 $(M-H_2O)^+$
HRMS: calcd for $C_{19}H_{33}O^{79}Br$: 356.1716
found : 356.1715

12 +  ⟶⟶ 20epi D5 (34a)

12 17mg (0.048mmol) を toluene 0.3mlに溶かし Et3N 0.45mlを加える. (Ar) (dba)3Pd2·CHCl3 1.9mg (0.03eq). Ph3P 2.5mg (0.3eq.) を加え rtでかくはんしつつ A環部 13mg (0.034mmol) in toluene (150μl + 50μl)を加える. 赤黒い溶液を rtで10minかくはんすると黄色溶液となる. 120°Cの oil bath上 2.5hr反応させる. 反応液を3か. ショートカラム (SiO2, EA = n-hex = 1:3)に付し 黄色oilを得る. (精製せずに次の反応へ.)

ホゴ体を MeOH 1mlにとかし CSA 11mg (0.047mmol)を加えて Ar下 rtで overnightかくはん. MeOHを溜去し 水を加え EA抽出. 有キ層を あつめて brineで洗い. MgSO4上 脱水 3かエバポレート. シリカゲルカラム (EA : n-hex = 1:1) にて精製. 無色結晶 9.3mg (y. 63%)を得る.

〈HPLCによる精製〉

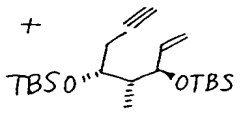
カラム: LiChrosorb RP-18 (7μm), 10×250, No. 301291
溶媒: Acetonitrile : 水 = 70 : 30
Recyclerをつけて 流速 7.0ml/min

UV (EtOH) : λmax 266nm
λmin 226nm
$\frac{A_{\lambda min}}{A_{\lambda max}} = 0.57$

^{1}H-NMR ($CDCl_3$-D_2O / TMS / 400MHz) δ 0.53 (3H, s) 0.85 (3H, d J = 6.7Hz) 1.08 (3H, d, J = 6.8Hz) 1.21 (6H, s) 1.12—2.04 (19H, m) 2.23 (1H, dd, J = 7.9Hz, 13.4Hz) 2.67 (4.0Hz, 13.4Hz) 2.83 (1H, m) 3.83 (1H, ddd, J = 7.9, 4.4, 4.0Hz) 4.29 (1H, d, J = 3.3Hz) 5.01 (1H, d, J = 1.8Hz) 5.28 (1H, m) 6.01 (1H, d, J = 11.3Hz) 6.39 (1H, d, J = 11.3Hz)

MS : 430 (M^+), 412 ($(M-H_2O)^+$), 394 ($(M-2H_2O)^+$)

HRMS : calcd for $C_{28}H_{46}O_3$: 430.3447
found : 430.3443

12 +  ⟶⟶ 20epi Aa (346)

12 15mg (0.042mmol) を toluene 0.3ml に溶かし Et3N 0.45ml を加える (Ar下) (dba)3Pd2·CHCl3 1.7mg, Ph3P 2.5mg を加え rtで かくはんしつつ A環部 13mg (0.034mmol) in toluene (150μl+50μl) を加え 10min かくはん. 120℃の oil bath上 4hr 反応させる. 反応液を セライトろかし, ショートカラム (EA:hex=1:3, SiO2) に付し. 黄色oilを得る.

ホゴ体を MeOH 1mlにとかし CSA 11mg (0.047mmol) を加えて Ar下 rtで overnight かくはん MeOHを溜去し. 水を加え EA抽出. 有機層を brineで洗い MgSO4上脱水 ろか. エバポレート. シリカゲルカラムにて (EA:n-hex=1:1) 精製後 無色結晶 4.5mg (y 31%) を得る.

<HPLCによる精製>
20epi Dsと同様の条件

UV (EtOH): λmax 263nm
λmin 228nm
$\frac{A\lambda_{min}}{A\lambda_{max}} = 0.55$

^{1}H-NMR (CDCl3-D2O/TMS/400MHz) δ 0.55 (3H, s) 0.85 (3H, d, J=6.4Hz) 1.15 (3H, d, J=6.7Hz) 1.21 (6H, s) 1.17−2.01 (19H, m) 2.42 (1H, dd, J=13.9, 4.9Hz) 2.52 (1H, d, J=13.9Hz) 2.82 (1H, dd, J=11.9Hz, 4.0Hz) 3.99−4.04 (1H+1H, m) 5.02 (1H, t, J=1.8Hz) 5.37 (1H, t, J=1.8Hz) 6.03 (1H, d, J=11.3Hz) 6.35 (1H, d, J=11.3Hz)

MS: 430 (M+), 412 (M−H2O)+, 394 (M−2H2O)+

HRMS: calcd for C28H46O3: 430.3447
found 430.3441

English translation

Experimental Seminar No. 3 Toshie Fujishima

● Synthesis of 2-methyl-20epi 1α,25(OH)2VD3 derivatives

実験セミナー No. 3 藤島 利江

◎ 2-methyl-20epi 1α,25(OH)2 VD3 誘導体の合成

◎ 1α,25(OH)2 VD3のA環部の合成法

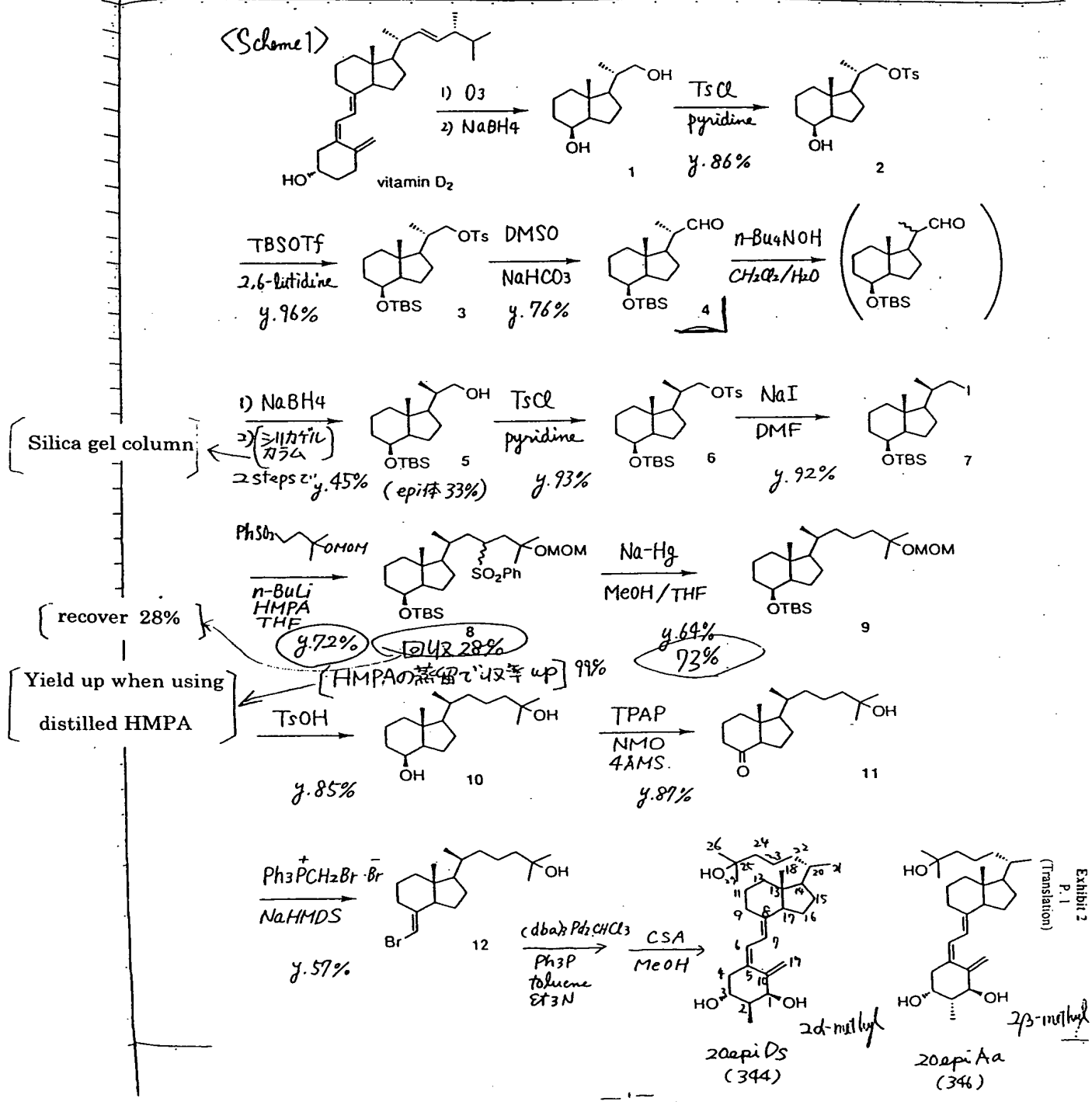


Exhibit 2
P. 1
(Translation)

Experiment of L...ine Thymus VDR binding affinity

Make diluted solution series by concentration preparation of 1α,25(OH)2VD3. #344, #346 according to λmax ε = 18000.

〈Bovine Thymus VDR への結合実験〉

① リン酸カリ buffer
phosphate-potassium buffer

K_2HPO_4	0.05M	pH 7.4
KH_2PO_4	0.05M	
KCl	0.3M	
DTT	5mM	

② 1α25(OH)$_2$VD3, #344, #346 と λmax の ε=18000 を用いて濃度調製し希釈系列を作成する.

ウシ胸腺ビタミンDレセプターはヤマサ醤油株式会社より購入し(lot.110431) 1アンプル (約25mg) を0.05Mリン酸0.5Mカリウムバッファー (pH 7.4) 55 mlに溶解した。ビタミンD誘導体のエタノール溶液50 µlとレセプター溶液500 µlを室温で1時間プレインキュベートした後、1α,25-(OH)$_2$[^{3}H]VD$_3$溶液50 µlを最終濃度0.1nMとなるように加えて4℃で一晩インキュベートした。結合と非結合の1α,25-(OH)$_2$[^{3}H]VD$_3$はデキストラン-コーテド-チャコール処理して遠心分離し、上澄に液シンカクテル(ACS-II)を加えて放射活性をカウントした。

free の drug が DCC にくっついて遠沈する

ビタミンD誘導体の活性は50%結合阻害する濃度を1α,25-(OH)$_2$VD$_3$を100としたときの比で表し評価した。

The content (about 25 mg) of an ample of a Bovine Thymus Vitamin D receptor (lot. 110431), which was purchased from YAMASA SYOUYU KABUSHIKIGAISYA, was dissolved in 55 ml of a 0.05 M phosphate 0.5 M potassium buffer (pH 7.4). After pre-incubation of 50 µl of ethanol solution of Vitamin D derivative with 500 µl of receptor solution for 1 hr at room temperature, 50 µl of 1α,25-(OH)2[3H]VD3 solution was added to the pre-incubation mixture so that the final concentration became 0.1 nM and the mixture was incubated overnight at 4℃. Both of the bound and non-bound (free drug is precipitated by sticking with DCC) 1α,25-(OH)2[3H]VD3 in the mixture was centrifuged after treatment of dextran coated charcoal, liquid scintillation cocktail (ACS-II) was added to the supernatant, and the radioactivity of the resultant mixture was measured.

The binding affinity of a compound to be tested for the Vitamin D receptor was expressed by a relative intensity ratio based on 100 for 1α,25-(OH)2[3H]VD3 by determining the concentration which inhibits the binding of the hot by 50%.

Exhibit 2
P. 2
(Translation)

cf. 〔20epi 1α,25(OH)$_2$VD3 の VDR への結合活性〕
- chicken intestine VDR 120
- bovine thymus VDR 500

Binding affinity of 20-epi 1α,25-(OH)2VD3 to VDR

Biochemical Pharm
47(6) 987-(19
42(8) 1569-7190

7→8 (#323)

側鎖部 sulfone 980mg (3eq) in dry THF (1.5ml)を Ar雰囲気下. HMPA 1.5ml (7eq)を加え一様とした後. −78℃に冷却した。 n-BuLi (1.6M in n-hexane) 2.3ml (3eq)を滴下し −78℃で 20minかくはん後 ヨード体 7 525mg (1.20mmol) in dry THF (2+洗い込み1ml)を滴下. −78℃で1hrかくはん後 反応液に sat NH4Clを加えて EA抽出. 有機層をあわせて brineで洗い. MgSO4上 脱水. ろか エバポレート シリカゲルカラム (EA:n-hex=1:8)にて精製し 無色oil 8 503mg (y.72%)を得ると共に1145mgの原料を回収(28%)

Side chain sulfone 980 mg (3 eq) in dry THF (1.5 ml) was added to HMPA 1.5 ml (7 eq) under Ar atmosphere and the mixture was cooled to −78℃ after make the mixture homogeneous. n-BuLi (1.6 M in n-hexane) 2.3 ml (3 eq) was added dropwise to the mixture and stirred for 20 min at −78 ℃. Iodo form 7 525 mg (1.20 mmol) in dry THF (2 + rinse 1 ml) was dropwise added to the mixture and stirred for 1 hr at −78 ℃. Sat. NH4Cl was added to the mixture and the resultant mixture was extracted with EA. The extract was combined with organic phase and this solution was washed with brine, dried over MgSO4, filtrated, and evaporated. The residue was purified by silica gel column chromatography (EA:n-hex = 1:8), 503 mg (y. 72%) of colorless oil 8 was obtained with 145 mg of the starting material 7 (28%) was recovered.

8→9 (#310)

8 165mg (0.28mmol)を dry THF 3ml. dry MeOH 3mlにとかし Na2HPO4 3.0g. 5% Na-Hg 9.8gを加えて Ar下 rtでかくはん overnight. 反応液を etherで希釈し セライトろか. 有機層を brineで洗い. MgSO4上脱水 ろか エバポレート シリカゲル カラム (EA=n-hex=1:9)にて精製 9 無色oil 80mg (y.64%)を 得ると共に原料 11mg (7%)を回収.

8 165 mg (0.28 mmol) was dissolved in dry THF 3 ml and dry MeOH 3 ml, Na2HPO4 3.0 g, 5% Na-Hg 9.8 g was added to the mixture and stirred overnight under Ar atmosphere at rt. The reaction mixture was diluted with ether and the resultant mixture was filtered through celite. The filtrate organic phase was washed with brine, dried over MgSO4, filtrated, and evaporated. The residue was purified by silica gel column chromatography (EA:n-hex = 1:9), 80 (y. 64%) mg of colorless oil 9 was obtained with 11 mg (7%) of the starting material was recovered.

9 → 10 (#316)

ホゴ体 9 80mg (0.18 mmol) を MeOH 3 ml に溶かし. TsOH·H2O 174mg (0.91 mmol) を加えて rt かくはん overnight. 反応液から MeOH を エバポレートし, シリカゲルカラム (EA : n-hex = 1 : 2) にて精製. 無色 oil 43mg (y. 85%) を得る.

The protected form 9 80 mg (0.18 mmol) was dissolved in MeOH 3 ml, TsCl·H2O 174 mg (0.91 mmol) was added to the mixture and stirred overnight at rt. MeOH was evaporated from the reaction mixture and the residue was purified by silica gel column chromatography (EA:n-hex = 1:2), 43 mg (y. 85%) of colorless oil was obtained.

10 → 11 (#326)

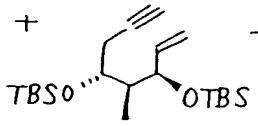
アルコール 10 117mg (0.41 mmol) dry CH_2Cl_2 (10 ml), 4ÅMS 30 mg を Ar 下 rt で 5分間かくはんする. TPAP 84 mg (0.24 mmol) を加えて 1 hr 20 min 後 反応液を small pad of silica gel 上 ろかし. エバポレート. シリカゲル カラム (EA : n-hex = 1 : 1) にて精製. 100mg (y. 87%) を得る.

The alcohol 10 117 mg (0.41 mmol) was dissolved in CH2Cl2 (10 ml), 4ÅMS 30 mg was added to the mixture and stirred for 5 min under Ar atmosphere at rt. TPAP 84 mg (0.24 mmol) was added to the mixture and the resultant mixture was filtered through small pad of silica gel after 1 hr 20 min. The filtrate was evaporated and the residue was purified by silica gel column chromatography (EA:n-hex = 1:1), 100 mg (y. 87%) was obtained.

11 → 12 (#334)

(bromomethyl)triphenyl phosphonium bromide 389 mg (5 eq) in dry THF (1.5 ml) を Ar 下 −60℃ に冷却し 1.0 M NaHMDS 0.86 ml (4.8 eq) を加え −60℃ で 1 hr 反応させた後. 11 50mg (0.18 mmol) in dry THF (1.5 ml) に transfer する. −60℃ → 0℃ → rt へと昇温し 1 hr 反応させた. 反応液に n-ヘキサンを加え セライトろかし ろ液をエバポレートして シリカゲルカラム (EA : n-hex = 1 : 8 → 1 : 3) にて精製. 12 36 mg (y. 56%) の淡黄 oil を得る.

(Bromomethyl)triphenyl phosphonium bromide 389 mg (5 eq) in dry THF (1.5 ml) was cooled to −60℃ under Ar atmosphere and 1.0 M NaHMDS 0.86 ml (4.8 eq) was added to the mixture. The resultant mixture was reacted for 1 hr at −60℃ and the mixture was transferred to 11 50 mg (0.18 mmol) in dry THF (1.5 ml). The reaction mixture was reacted for 1 hr under the reaction temperature was warmed −60℃ → 0℃ → rt. n-Hexane was added to the reaction mixture and filtered through celite. The filtrate was evaporated and the residue was purified by silica gel column chromatography (EA:n-hex = 1:8 → 1:3), 36 mg (y. 56%) of pale yellow oil 12 was obtained.

12 +  ⟶⟶ 20epi Ds (344)

12 17mg (0.048mmol) を toluene 0.3mlに溶かし Et3N 0.45mlを加える. (Ar下 (dba)3Pd2·CHCl3 1.9mg (0.03eq). Ph3P 2.5mg (0.3eq.) を加え rtでかくはんしつつ A環部 13mg (0.034mmol) in toluene (150μl + 50μl) を加える. 赤黒い溶液を rtで10minかくはんすると黄色溶液となる. 120℃の oil bath上 2.5hr反応させる反応液をろか, ショートカラム (SiO2, EA=n-hex=1:3) に付し 黄色oilを得る. (精製せずに次の反応へ.)

ホゴ体を MeOH 1mlにとかし CSA 11mg (0.047mmol) を加えて Ar下 rtで overnightかくはん. MeOHを留去し 水を加え EA抽出. 有キ層をあつめて brineで洗い. MgSO4上 脱水 ろかエバポレート. シリカゲルカラム (EA:n-hex=1:1) にて精製. 無色結晶 9.3mg (y.63%) を得る.

<HPLCによる精製>

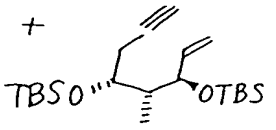
カラム: LiChrosorb RP-18 (7μm), 10×250, No.301291
溶媒: Acetonitrile : 水 = 70 : 30
recyclerをつけて 流速7.0ml/min

12 17 mg (0.048 mmol) was dissolved in toluene 0.3 ml, Et3N 0.45 ml was added to the mixture (under Ar atmosphere). (dba)3Pd2·CHCl3 1.9 mg (0.03 eq), Ph3P 2.5 mg (0.3 eq) were added to the mixture. A-ring part 13 mg (0.034 mmol) in toluene (150 μl + 50 μl) was added to the mixture under stirring of the mixture at rt. The resultant red-black colored solution was changed to yellow solution during stirring for 10 min at rt. The resultant mixture was reacted for 2.5 hr in an oil bath at 120 ℃. The reaction mixture was filtered, the filtrate was evaporated, and the residue was treated with short column chromatography (SiO2, EA:n-hex = 1:3), yellow oil was obtained. (The next reaction was carried out without purification)

The protected form was dissolved in MeOH 1.0 ml, CSA 11 mg (0.047 mmol) was added to the mixture, and stirred overnight at rt under Ar atmosphere. MeOH was evaporated, water was added to the resultant residue and extracted with EA. The combined organic phase was washed with brine, dried over MgSO4, filtrated, and evaporated. The residue was purified by silica gel column chromatography (EA:n-hex = 1:1), 9.3 mg (y. 63%) of colorless crystal was obtained.

<Purification by HPLC>

column: LiChrosorb RP-18 (7 μm), 10 x 250, No. 301291
solvent: Acetnitrile : water = 70 : 30
flow rate 7.0 ml / min with recycler

12 +  ⟶⟶ 20epi Aa (346)

12 15 mg (0.042 mmol) を toluene 0.3 ml に溶かし Et3N 0.45 ml を加える (Ar下) (dba)3Pd2·CHCl3 1.7 mg, Ph3P 2.5 mg を加え rt で かくはんしつつ A環部 13 mg (0.034 mmol) in toluene (150 μl + 50 μl) を加え 10 min かくはん. 120℃の oil bath上 4hr 反応させる. 反応液を セライトろかし, ショートカラム (EA:n-hex = 1:3, SiO2) に付し. 黄色oilを得る.

ホゴ体を MeOH 1 ml にとかし CSA 11 mg (0.047 mmol) を加えて Ar下 rt で overnight かくはん MeOHを溜去し. 氷を加え EA抽出. 有機層を brine で洗い MgSO4上脱水ろか. エバポレート. シリカゲルカラムにて (EA:n-hex=1:1) 精製後 無色結晶 4.5 mg (y 31%) を得る.

<HPLCによる精製>
20epi Dsと同様の条件

12 15 mg (0.042 mmol) was dissolved in toluene 0.3 ml, Et3N 0.45 ml was added to the mixture (under Ar atmosphere). (dba)3Pd2·CHCl3 1.7 mg, Ph3P 2.5 mg were added to the mixture. A-ring part 13 mg (0.034 mmol) in toluene (150 μl + 50 μl) was added to the mixture under stirring at rt and the mixture was stirred for 10 min. The resultant mixture was reacted for 4 hr in an oil bath at 120 ℃. The reaction mixture was filtered through celite, the filtrate was evaporated and the residue was treated with short column chromatography (SiO2, EA:n-hex = 1:3), yellow oil was obtained.

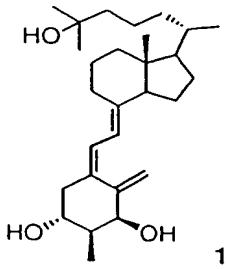
The protected form was dissolved in MeOH 1.0 ml, CSA 11 mg (0.047 mmol) was added to the mixture, and stirred overnight at rt under Ar atmosphere. MeOH was evaporated, water was added to the resultant residue and extracted with EA. The combined organic phase was washed with brine, dried over MgSO4, filtrated, and evaporated. The residue was purified by silica gel column chromatography (EA:n-hex = 1:1), 4.5 mg (y. 31%) of colorless crystal was obtained.

<Purification by HPLC>

same condition as 20 epi Ds.

SYNTHESIS AND BIOLOGICAL ACTIVITIES OF 2-METHYL-20-EPI ANALOGUES OF 1α,25-DIHYDROXYVITAMIN D_3. T. Fujishima, Z.-P. Liu, K. Konno and H. Takayama Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-01, JAPAN.

Active conformations of the A-ring of 1α,25-dihydroxyvitamin D_3 is still unclear. In order to investigate the conformation-activity relationship of the A-ring portion, we have synthesized the 2-methyl analogues of 1α,25-dihydroxyvitamin D_3, demonstrating that the introduction of the 2-methyl group elevates the affinity to the nuclear receptor (VDR) in some cases. In the present work, we designed and synthesized 2-methyl-20-epi analogues of 1α,25-dihydroxyvitamin D_3. The binding affinities of the synthesized compounds were preliminarily tested using the bovine thymus vitamin D receptor. The 2α-methyl-20-epi analogue (1) exhibited about ten-fold higher potency than 1α,25-dihydroxyvitamin D_3, whereas the 2β-methyl-20-epi analogue (2) had similar activity to 1α,25-dihydroxyvitamin D_3.



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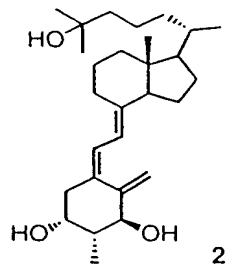

2

TENTH WORKSHOP ON VITAMIN D -- Strasbourg, France - May 24 - 29, 1997
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- ✓ (b) Chemistry: structure/function (interface with biology)
- ___ (c) Vitamin D/Analogs metabolism & catabolism
- ___ (d) $1,25(OH)_2D_3$ Receptors (biochemistry & molecular biology)
- ___ (e) Gene regulation by vitamin D steroids
- ___ (f) Retinoids & $1,25(OH)_2D_3$
- ___ (g) Rapid/Non-genomic actions
- ___ (h) Cell differentiation/Proliferation
- ___ (i) Transplantation/Immunology
- ___ (j) Brain, neural tissue
- ___ (k) Intestinal & renal actions
- ___ (l) Skin (actions)
- ___ (m) Bone, cartilage
- ___ (n) Muscle
- ___ (o) Calbindins-D and other Ca^{2+} binding protein
- ___ (p) Biological actions of vitamin D metabolites (other)
- ___ (q) Development & vitamin D
- ___ (r) Hormone secretion
- ___ (s) Hydroxylases (biochemistry & regulation)
- ___ (t) Evolutionary aspects
- ___ (u) Vitamin D-binding protein (DBP)
- ___ (v) Assay methodology (vitamin D & metabolites)
- ___ (w) Other (basic science topics)

Clinical Topics and Vitamin D
- ___ (x) $1,25(OH)_2D_3$ Receptor (VDR) polymorphisms
- ___ (y) Rickets & osteomalacia
- ___ (z) Osteoporosis
- ___ (za) Renal osteodystrophy
- ___ (zb) Cancer
- ___ (zc) Neonatology/Pregnancy/Development
- ___ (zd) Aging
- ___ (ze) Dermatology
- ___ (zf) Nutritional aspects
- ___ (zg) Other (clinical topics)

Type name and mailing address of submitting author:
Hiroaki Takayama
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Tel.: (81) 426-85-3713 FAX: (81) 426-85-3714
Signature of submitting author: Hiroaki Takayama

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3. Be certain the TITLE IS COMPLETELY CAPITALIZED. The title should be followed by authors' names (initials or first name must precede the last name). Authors' names shall be continuously underlined. Department, institutional affiliation, city, country and postal zip code are required (do not include street address).

4. SINGLE SPACE all typing in the abstract. The text should be a single paragraph which starts with a three-space indentation.

5. When using abbreviations for compounds, spell them out in full when first mentioned, followed by the abbreviation in parentheses. Do not use "vitamin D" or "V-D" as a substitute/synonym for "$1\alpha,25(OH)_2D_3$"; they are chemically different compounds.

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   Department of Biochemistry
   Boyce Hall, Room 5456
   University of California
   Riverside, CA 92521 USA

9. This abstract must be received by **Monday, February 10, 1997.** FAX COPIES WILL NOT BE ACCEPTED.

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   deadline

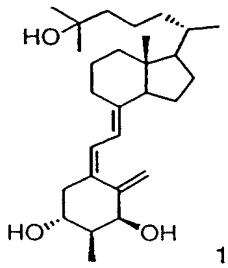
Example:

EXPRESSION OF THE CALBINDIN-D_{28K} GENE IS ACC
CHANGES IN CHROMATIN STRUCTURE. L. Brown and I
Biochemistry, University of Washington, Seattle, WA, USA 981-

The chromatin structure of the chicken calbindin-D_{28K} and flanking DNA was studied in different chicken tissues. Expression of eukaryotic genes is accompanied by changes in the local structural organization of chromatin.

SYNTHESIS AND BIOLOGICAL ACTIVITIES OF 2-METHYL-20-EPI ANALOGUES OF 1α,25-DIHYDROXYVITAMIN D_3. T. Fujishima, Z.-P. Liu, K. Konno and H. Takayama Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-01, JAPAN.

Active conformation of the A-ring of 1α,25-dihydroxyvitamin D_3 is still unclear. In order to investigate the conformation-activity relationship of the A-ring portion, we have synthesized the 2-methyl analogues of 1α,25-dihydroxyvitamin D_3, demonstrating that the introduction of the 2-methyl group elevates the affinity to the nuclear receptor (VDR) in some cases (preceding abstract). In the present work, we designed and synthesized 2-methyl-20-epi analogues of 1α,25-dihydroxyvitamin D_3. The binding affinities of the synthesized compounds were preliminarily tested using the bovine thymus vitamin D receptor. The 2α-methyl-20-epi analogue (1) exhibited about ten-fold higher potency than 1α,25-dihydroxyvitamin D_3, whereas the 2β-methyl-20-epi analogue (2) had similar activity to 1α,25-dihydroxyvitamin D_3. Synthesis and biological evaluation of other stereoisomers of the 20-epi analogues will be presented.



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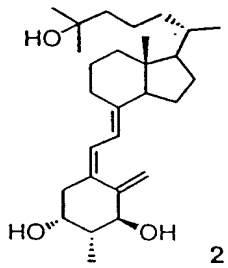

2

TENTH WORKSHOP ON VITAMIN D -- Strasbourg, France - May 24 - 29, 1997
ABSTRACT REPRODUCTION FORM (Instructions for preparing the abstract are given on the reverse side.)

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For an abstract submitted as a Free Communication, indicate below the one session you prefer for scheduling this abstract:

Basic Science Topics and Vitamin D

- ___ (a) Metabolites & analogs: pure chemistry
- ✓ (b) Chemistry: structure/function (interface with biology)
- ___ (c) Vitamin D/Analogs metabolism & catabolism
- ___ (d) $1,25(OH)_2D_3$ Receptors (biochemistry & molecular biology)
- ___ (e) Gene regulation by vitamin D steroids
- ___ (f) Retinoids & $1,25(OH)_2D_3$
- ___ (g) Rapid/Non-genomic actions
- ___ (h) Cell differentiation/Proliferation
- ___ (i) Transplantation/Immunology
- ___ (j) Brain, neural tissue
- ___ (k) Intestinal & renal actions
- ___ (l) Skin (actions)
- ___ (m) Bone, cartilage
- ___ (n) Muscle
- ___ (o) Calbindins-D and other Ca^{2+} binding protein
- ___ (p) Biological actions of vitamin D metabolites (other)
- ___ (q) Development & vitamin D
- ___ (r) Hormone secretion
- ___ (s) Hydroxylases (biochemistry & regulation)
- ___ (t) Evolutionary aspects
- ___ (u) Vitamin D-binding protein (DBP)
- ___ (v) Assay methodology (vitamin D & metabolites)
- ___ (w) Other (basic science topics)

Clinical Topics and Vitamin D

- ___ (x) $1,25(OH)_2D_3$ Receptor (VDR) polymorphisms
- ___ (y) Rickets & osteomalacia
- ___ (z) Osteoporosis
- ___ (za) Renal osteodystrophy
- ___ (zb) Cancer
- ___ (zc) Neonatology/Pregnancy/Development
- ___ (zd) Aging
- ___ (ze) Dermatology
- ___ (zf) Nutritional aspects
- ___ (zg) Other (clinical topics)

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